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(71)(72) Applicants and Inventors: CRAMER, Steven, M. [US/US]; 783 Trottingham Drive, Schenectady, NY 12309 (US). MOORE, James, A. [US/US]; 3 Audrey Lane, R.D. #4, Troy, NY 12180 (US).

(72) Inventors: KUNDU, Amitava; 77 Eagle Street, Troy, NY 12180 (US). LI, Yufei; Institute of Chemistry, Academia Sinica, Beijin (CN). JAYARAMAN, Guhan; A-4 57 Burkitt Road, T. Nagar, Madras, 600017 (IN).

(74) Agents: HANSEN, Philip, E. et al.; Heslin & Rothenberg, 5 Columbia Circle, Albany, NY 12203-5160 (US).

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(54) Title: DISPLACEMENT CHROMATOGRAPHY OF PROTEINS USING LOW MOLECULAR WEIGHT DISPLACERS

(57) Abstract

A method for the purification of proteins by displacement chromatography on ion exchange media using molecular weight displacers is disclosed. Several classes of low molecular weight, charged species are exemplified, including aminoacids, peptides, antibiotics and dendrimeric polymers. Novel compounds useful as displacers are dendrimers of formula (I), wherein R^1 is lower alkyl, n is 2 to 6, and X is a common counter anion and similar dendritic polymers based thereon.
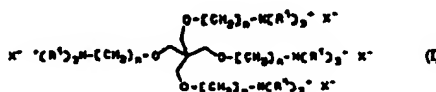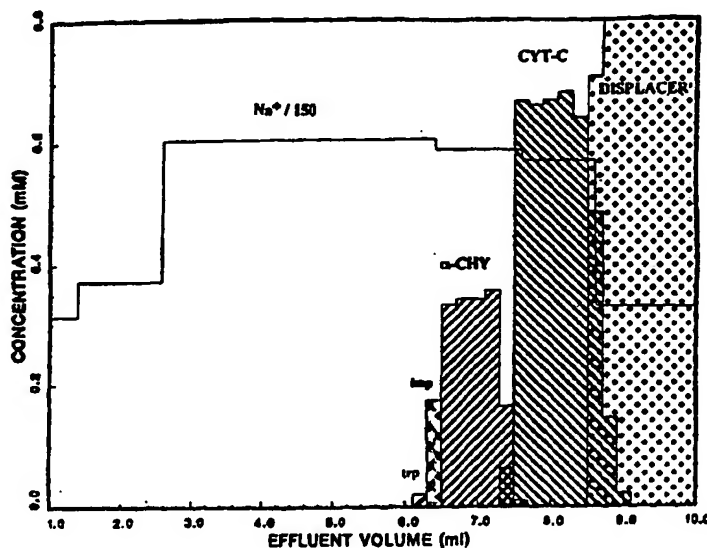
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DISPLACEMENT CHROMATOGRAPHY OF PROTEINS USING LOW MOLECULAR WEIGHT DISPLACERS

Statement As To Rights Under Federally Sponsored Research

Field of the Invention

10   This invention relates to the displacement chromatography of proteins using low molecular weight displacers and to a novel class of dendritic polymer based polyelectrolytes useful for chromatography of proteins.

Background of the Invention

15

        The displacement mode of chromatography was first recognized in 1906 by Tswett, who noted that sample displacement occurred under conditions of overloaded elution chromatography. In 1943, Tiselius
20   developed the classifications of frontal chromatography, elution chromatography , and displacement chromatography. Since that time most developments and applications, particularly those in analytical chromatography, have taken place in the
25   area of elution chromatography, and indeed the term chromatography without further qualification usually refers to elution chromatography. Nonetheless, while the theory and practice of elution chromatography has dominated the literature for the past fifty years,

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the theory and practice of displacement chromatography has occupied a small niche in chromatographic science.

5   The two types of chromatography, elution and displacement, are readily distinguished both in theory and in practice. In elution chromatography, a solution of the sample to be purified (in the case of the present invention, a protein) is applied to a stationary phase, commonly in a column. The mobile
10   phase is chosen such that the sample is neither irreversibly adsorbed nor totally unadsorbed, but rather binds reversibly. As the mobile phase is flowed over the stationary phase, an equilibrium is established between the mobile phase and the
15   stationary phase whereby, depending upon the affinity for the stationary phase, the sample passes along the column at a speed which reflects its affinity relative to other components that may occur in the original sample. The differential migration process
20   is outlined schematically Fig. 1, and a typical chromatogram is shown in Figure 2. Of particular note in standard elution chromatography is the fact that the eluting solvent front, or zero column volume in isocratic elution, <u>always</u> precedes the sample off
25   the column.

       A modification and extension of isocratic elution chromatography is found in step gradient chromatography wherein a series of eluents of varying composition are passed over the stationary phase. In
30   ion exchange chromatography, step changes in the mobile phase salt concentration and/or pH are employed to elute or desorb the proteins.

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        Displacement chromatography is fundamentally
    different from desorption chromatography (e.g.,
    affinity chromatography, step gradient
    chromatography). The displacer, having an affinity
5   higher than any of the feed components, competes
    effectively for the adsorption sites on the
    stationary phase. An important distinction between
    displacement and desorption is that the displacer
    front always remains <u>behind</u> the adjacent feed zones
10 in the displacement train, while desorbents (e.g.,
    salt, organic modifiers) move <u>through</u> the feed zones.
    The implications of this distinction are quite
    significant in that displacement chromatography can
    potentially concentrate and purify components from
15 mixtures having low separation factors while in the
    case of desorption chromatography, relatively large
    separation factors are generally required to give
    satisfactory resolution.

        In displacement chromatography the eluent, (i.e.
20 the displacer) has a higher affinity for the
    stationary phase than does any of the components in
    the mixture to be separated. This is in contrast to
    elution chromatography, where the eluent usually has
    a lower affinity. The key operational feature which
25 distinguishes displacement chromatography from
    elution or desorption chromatography is the use of a
    displacer molecule. In displacement chromatography,
    the column is first equilibrated with a carrier
    solvent under conditions in which the components to
30 be separated all have a relatively high affinity for
    the stationary phase. A large volume of dilute feed
    mixture can be loaded onto the column and individual
    components will adsorb to the stationary phase. That

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is, they distribute from the feed solution onto the
stationary phase, and remain there. If all the
components are to be resolved by displacement, the
carrier solvent emerges from the column containing no
5   sample. The sample now resides on the stationary
phase and the position of each component on the
column is correlated with its relative affinity for
the stationary phase. Conceptually, one can imagine
each molecule of the component with the highest
10   affinity for the stationary phase displacing a
molecule of a component having a lower affinity at a
site on the stationary phase so that the individual
components will ultimately be arranged on the column
in sequence from highest to lowest affinity.

15   It will sometimes be advantageous to allow some
of the components to pass through the column with the
carrier solvent; in this case only the retained feed
components will be resolved by displacement
chromatography.

20   Once the sample is loaded on the column, a
displacer solution is introduced. The displacer
solution comprises a displacer in a suitable solvent.
The displacer is selected such that it has a higher
affinity for the stationary phase than does any of
25   the feed components. Assuming that the displacer and
mobile phase are appropriately chosen, the product
components exit the column as adjacent squarewave
zones of highly concentrated pure material in the
order of increasing affinity of absorption. This is
30   shown schematically in Fig. 3. Following the zones
of purified components, the displacer emerges from
the column. A typical chromatogram from a

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displacement chromatography is shown in Fig. 4. It
is readily distinguished from the chromatogram of
elution chromatography shown in Fig. 2 by virtue of
the fact that the displacer <u>follows</u> the sample and
5   that the feed components exit the column as adjacent
"square wave" zones of highly concentrated pure
material. Finally, after the breakthrough of the
displacer, the column is regenerated by desorbing the
displacer from the stationary phase to allow the next
10   cycle of operation.

Displacement chromatography has some
particularly advantageous characteristics for process
scale chromatography of biological macromolecules
such as proteins. First, and probably most
15   significantly, displacement chromatography can
achieve product separation and concentration in a
single step. By comparison, isocratic elution
chromatography results in product dilution during
separation. Second, since the displacement process
20   operates in the nonlinear region of the equilibrium
isotherm, high column loadings are possible. This
allows much better column utilization than elution
chromatography. Third, column development *per se*
requires less solvent than a comparable elution
25   process. Fourth, displacement chromatography can
concentrate and purify components from mixtures
having low separation factors, while relatively large
separation factors are required for satisfactory
resolution in desorption chromatography.

30   With all of these advantages, one might presume
that displacement chromatography would be widely
utilized. However, displacement chromatography, as

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it is traditionally known, has a number of drawbacks vis-à-vis elution chromatography for the purification of proteins. The term "protein", as commonly understood in the art and as used herein, refers to
5 polypeptides of 10 kDa molecular weight or more; according to this convention, polypeptides of molecular weight below 10 kDa are commonly referred to as oligopeptides. Two of the major problems are (1) regeneration of the column and (2) the presence
10 of displacer in some of the purified fractions.

Since the displacement process uses a high affinity compound as the displacer, the time for regeneration and re-equilibration can be long compared to elution chromatography. Furthermore,
15 relatively large amounts of solvent are often required during regeneration, effectively reducing any advantage over elution chromatography in solvent consumption.

The second problem, that of contamination by the
20 displacer, has arisen because a common characteristic of displacers used in protein separations has been their relatively high molecular weight. Heretofore the art has taught the use of high molecular weight polyelectrolytes to displace proteins on the
25 assumption that (as explained below) it is necessary to have a large polyelectrolyte in order to ensure a higher binding coefficient than the protein that is to be displaced. High molecular weight displacers exhibit both of the disadvantages enumerated above:
30 they bind tightly to the stationary phase and therefore require heroic conditions for regenerating the column, and traces of the displacer that may

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contaminate the product fraction are difficult to
remove.

    Therefore, it would be advantageous to have a
class of displacers that did not require extensive
5 regeneration of the column and that could be readily
removed from the product protein. There is one
example in the art known to applicants of an attempt
to use 2 kilodalton poly(vinylsulfonic acid) on
polyethyleneimine-coated weak anion exchange resin
10 for the separation of conalbumin from ovalbumin. The
experiment appears to have been successful in that
the two proteins were separated [See Jen and Pinto
Journal of Chromatography 519, 87-98 (1990)]. However
the separation appeared to have been effected by a
15 mixed mechanism of elution and displacement
chromatography, as discussed in a subsequent paper
[see Jen and Pinto Journal of Chromatographic Science
29, 478-484 (1991)] in which the authors abandoned
the poly(vinyl sulfate) displacers in favor of higher
20 molecular weight dextran sulfate. In this second
paper, Jen and Pinto demonstrate the superiority of
the larger dextran sulfate over the smaller polyvinyl
sulfate.

    In a subsequent article, Jen and Pinto [Reactive
25 Polymers 19, 145-161 (1993), p.147] provide a table
of all displacers used for the displacement
chromatography of proteins on ion exchange stationary
phases. In their discussion of the results, they
conclude, as before, that the 2kDa polyvinyl sulfate
30 partially displaces the second protein and elutes the
first.

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It has now been surprisingly found that several classes of charged species of very low molecular weight can function very efficiently as displacers for proteins in displacement chromatography.

5

Summary of the Invention

In one aspect, the invention relates to a method for purifying a protein, or several proteins, comprising loading the protein in a suitable mobile phase onto an ion exchange stationary phase and
10 displacing the protein from the stationary phase by displacement chromatography using a displacer of molecular weight less than 2000.

In one embodiment the stationary phase is a cation exchange resin and the displacer is a cationic
15 species; in another embodiment the stationary phase is an anion exchange resin and the displacer is an anionic species. In various preferred embodiments the displacer is a poly(quaternary ammonium) salt, or the displacer is an aminoacid ester, aminoacid amide, N-
20 acylaminoacid, peptide ester, peptide amide or N-acyl peptide, preferably lower alkyl esters or amides of lysine, arginine, N^α-acylated lysine, or N^α-acylated arginine. Lower alkyl refers to linear, branched or cyclic, saturated hydrocarbon residues of six or
25 fewer carbons. The displacer may also be a cationic or anionic antibiotic, or a dendritic polymer. When the displacer is a dendritic polymer, a preferred displacer is

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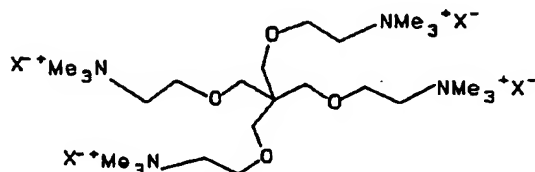
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wherein X⁻ is a counter anion, for example halogen,
sulfate, sulfonate, perchlorate, acetate, phosphate
or nitrate.

5   Generally, the displacer may be advantageously
selected from electrolytes whose characteristic
charge (ν) and equilibrium constant (K) are such that
when a coordinate system representing log K on the
ordinate and ν on the abscissa is created, a line

10   constructed from a point A on the ordinate axis
through a point defined by the K and the ν of the
displacer has a greater slope than a corresponding
line constructed from the same point A through a
point defined by the K and the ν of the protein to be

15   purified. The point A corresponds in value to the
slope of the displacer operating line (Δ) in the
system of interest. This will be explained in
greater detail below. Displacers of molecular weight
below 900 are particularly advantageous.

20   In another aspect the invention relates to a
method for purifying a protein comprising loading the
protein in a suitable loading solvent onto an ion
exchange stationary phase and displacing the protein
from the stationary phase by displacement

25   chromatography using a dendritic polyelectrolyte
displacer. The stationary phase can be a cation
exchange resin, in which case the polyelectrolyte
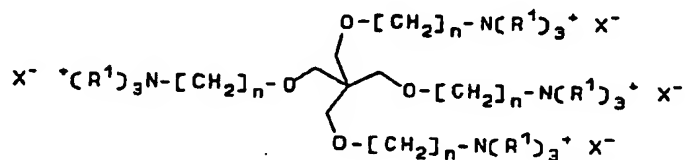will be a polycation, or the stationary phase can be

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an anion exchange resin, in which case the
polyelectrolyte will be a polyanion. Preferably, the
polyelectrolyte is a poly(quaternary ammonium) salt.
Another preferred dendritic polymer is

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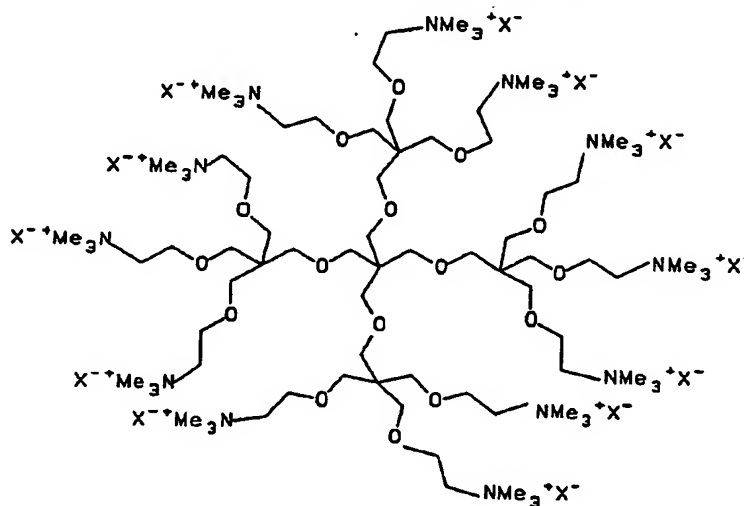
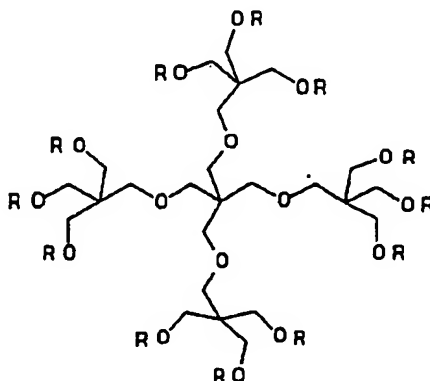In another aspect, the invention relates to compounds
of formula

and

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wherein R is $-(CH_2)_n-N(R^1)_3^+$ X^-, R^1 is lower alkyl, n is 2 to 6, and X is as before. The compounds are useful as displacers in displacement chromatography.

Brief Description of the Drawings

5   Fig. 1 is a schematic representation of a standard isocratic linear elution chromatography.

Fig. 2 is a typical chromatogram from elution chromatography.

Fig. 3 is a schematic representation of

10   displacement chromatography.

Fig. 4 is a typical chromatogram from displacement chromatography.

Fig. 5 is a plot of equilibrium constant (K) versus characteristic charge (v) for two proteins and

15   two displacers of the invention.

Figs. 6, 7 and 8 are chromatograms of proteins using displacers of the invention.

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Detailed Description of the Invention Including Preferred Embodiments

A better understanding of the surprising discovery that small molecules can be used
5   effectively as displacers in the chromatography of proteins is gained by briefly considering an improved mathematical model for displacement chromatography. Although this hypothetical construct is useful to rationalize the phenomenon, it is not intended to
10   limit the full breadth of the invention.

The steric mass action (SMA) ion exchange model developed by one of the inventors, unlike other models, explicitly accounts for steric effects in multicomponent protein equilibria and is able to
15   predict complex behavior in ion exchange displacement systems. A macromolecular solute like a protein or a polyelectrolyte is presumed to have a multi-point attachment on an ion-exchange surface and the number of interactions between the absorbent surface and a
20   single macromolecule is defined as the characteristic charge of the solute molecule. The characteristic charge of a solute is numerically equal to the number of salt counter-ions displaced by the solute from the ion-exchange surface upon adsorption. However, in
25   addition to the sites at which the polyelectrolyte actually interacts, a large solute macromolecule bound to an ion-exchange surface also sterically hinders the adsorption of macromolecules of similar size onto sites underneath and adjoining the bound
30   solute molecule. The number of sterically hindered salt counter-ions on the surface (per adsorbed solute molecule), unavailable for exchange with other solute

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molecules in the fluid phase is defined as the steric
factor of the adsorbed macromolecule. Earlier
treatments of mass action ion exchange equilibria
assumed that the binding of a macromolecule to an
5   adsorbent surface only affects a number of adsorbent
sites equal to its characteristic charge. In fact,
the steric shielding of the stationary phase sites
plays an important role in the non-linear adsorption
behavior of macromolecules in ion-exchange systems.

10   The stoichiometric exchange of a solute molecule
(protein or polyelectrolyte) and the exchangeable
salt counter-ions can be represented by:

$$C_i + v_i \overline{Q_s} \rightleftharpoons Q_i + v_i C_s \qquad (1)$$
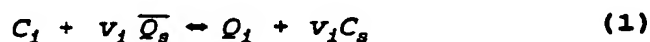
where C and Q are the mobile and stationary phase
concentrations; v_i is the characteristic charge of the
15   solute, and subscripts i and s refer to the solute
molecule and the salt counter-ion respectively. The
overbar denotes bound salt counter-ions available for
exchange with the solute macromolecule in solution.
The equilibrium constant, K_i, for the solute adsorbed
20   on the ion-exchange surface is given by:

$$K_i = \left(\frac{Q_i}{C_i}\right) \left(\frac{C_s}{\overline{Q_s}}\right)^{v_i} \qquad (2)$$

The equilibrium constant is a measure of the affinity
of the molecule. The electro-neutrality condition on
the stationary phase is given by the following
relation:

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$$\Lambda = \overline{Q_s} + (v_i + \sigma_i)\, Q_i \tag{3}$$

where σ_i is the steric factor of the displacer or protein.

Substituting equation 3 into equation 2 and re-arranging yields the following equilibrium relation

5   for a single protein or displacer:

$$C_i = \left(\frac{Q_i}{K_i}\right)\left(\frac{C_s}{\Lambda - (v_i + \sigma)\, Q_i}\right)^{v_i} \tag{4}$$

Thus, knowing the values of the mobile phase counter-ion concentration C_s, the column ion-bed capacity, Λ, and the model parameters for each component, one can easily generate a single component

10   isotherm from the implicit equation (4). The required model parameters for each component are: characteristic charge, v_i, steric factor, σ_i and equilibrium constant K_i. In order to employ this model for predicting displacement behavior, it is

15   necessary to determine model parameters for the proteins and the displacers.

Ion-bed capacity, Λ, can be measured *in-situ* using frontal chromatographic techniques [see Gadam et al., _J. Chromatog._ 630, 37-52 (1993)].

20   For protein molecules exhibiting significant salt-sensitive retention behavior under low to moderate salt concentrations in the mobile phase, linear elution chromatography can be employed to determine two of the three SMA model parameters

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(viz., characteristic charge and equilibrium constant) using well established relationships for ion-exchange systems [see Kopaciewicz et al., *J. Chromatog.* 266, 3 (1983)]. Linear elution
5   experiments are carried out at various mobile phase salt concentrations in order to determine the characteristic charge (v_i) and equilibrium constant (K_i) by the following equations:

$$\log k' = \log(\beta K_i \Lambda^{v_i}) - v_i \log C_s \qquad (5)$$

where, for a log k' v log C_s plot,
10   slope = $- v_i$; and intercept = log ($\beta K_i \Lambda^{v_i}$).

Having determined the characteristic charges and equilibrium constants for the proteins, the remaining SMA parameter, viz. steric factor, σ_i, for the proteins is determined independently from a single
15   non-linear frontal chromatographic experiment according to the expression:

$$\sigma_i = \frac{\beta}{C_{f_i}\Pi}\left(\Lambda - C_s\left(\frac{\Pi}{\beta K_i}\right)^{1/v_i}\right) - v_i \qquad (6)$$

where,

$$\Pi = \left(\frac{V_b}{V_o} - 1\right) \qquad (7)$$

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Many proteins exhibit significantly higher steric factors relative to their characteristic charge, which is not surprising in light of the conformational constraints in the protein molecules.
5   Once the SMA parameters are obtained for a given protein, the model can then be used to generate adsorption isotherms at any salt concentration.

While the determination of the characteristic charge and equilibrium constant from linear elution
10 data works well for moderately retained proteins, it is quite difficult to characterize high affinity displacers in this fashion. Frontal chromatography, on the other hand, is well suited for parameter estimations for these high affinity compounds. The
15 characteristic charge of the displacer, v_D, can be determined from the induced salt gradient using the following expression:

$$v_D = \frac{n_I}{n_D} = \frac{\Delta C_s}{C_D} \tag{8}$$

wherein n_I is the total amount of ions displaced, n_D is the number of moles of displacer adsorbed on the
20 stationary phase, C_D is the mobile phase concentration of polyelectrolyte displacer and ΔC_s is the step increase in the mobile phase counter-ion concentration upon displacer adsorption.

At sufficiently low mobile phase salt
25 concentration the displacer completely saturates the stationary phase material. Frontal experiments under these conditions can be employed to determine the steric factor, σ_D, from the following expression

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$$\sigma_D = \frac{\Lambda}{Q_D^{max}} - n_D \qquad (9)$$

where Λ is the ion bed capacity and Q_D^{max} is the maximum stationary phase capacity of the polyelectrolyte displacer. Alternatively, the steric factor could be determined by measuring, for example,
5   the sterically hindered sodium ions displaced by an ammonium front (analogous to bed-capacity measurement), n_2, as given by

$$\sigma_D = \frac{n_2}{n_D} \qquad (10)$$

The equilibrium constant for the ion-exchange process is defined by equation 2. Once the characteristic
10   charge and steric factor are measured independently as described above, a frontal experiment is employed for the determination of the equilibrium constant K_D. This experiment is performed under elevated mobile phase salt conditions where the solute does not
15   completely saturate the bed. The equilibrium constant is directly calculated from the breakthrough volume using the independently determined values of the characteristic charge (v_D) and steric factor (σ_D) by the expression

$$K_D = \frac{1}{\beta} \left(\frac{V_b}{V_o} - 1 \right) \left(\frac{C_S}{\Lambda - (v_D + \sigma_D) \frac{C_D}{\beta} \left(\frac{V_b}{V_o} - 1 \right)} \right)^{v_D} \qquad (11)$$

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where β is the column phase ratio and C_s is the
initial salt concentration in the carrier. Once the
characteristic charge, steric factor and equilibrium
constants are determined, the isotherms of the
5   proteins and polyelectrolytes can be simulated using
the SMA formalism described above.

According to the conventional wisdom based on
results observed with derivatized polysaccharide
displacers, a high molecular weight compound with a
10 relatively high characteristic charge and a high
steric factor to characteristic charge ratio is
needed for protein displacement chromatography.
There have been heretofore no clearly defined
criteria for selecting or determining the efficacy of
15 one displacer over another. Using the mathematical
model described above, it is now possible to predict
the elution order of the feed components as a
function of the characteristic charge and equilibrium
constant of each of the components, once the slope of
20 the displacer operating line is known.

The mathematical criterion for effective
displacement chromatography can be reconstructed as a
plot of log K_i vs. v_i (see Fig. 5). The elution order
in the isotachic displacement train can be then
25 graphically determined by constructing lines from the
point on the ordinate axis corresponding to the slope
of the displacer operating line, Δ,

$$\Delta \;=\; \frac{Q_d}{C_d} \;=\; K_{1_d}\left[\frac{\Lambda-(v_d+\sigma_d)\,Q_d}{(C_1)_d}\right] \qquad (12)$$

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through each of the points defined by the equilibrium
parameters (characteristic charge and equilibrium
constant) of the solutes. The order of elution of
the feed components corresponds to the counter-
5   clockwise order (i.e. increasing slopes) of these
"affinity" lines. In equation (12) $(C_1)_d$ is the
concentration of salt that the displacer encounters
(i.e. the carrier salt concentration), Q_d is the
concentration of the displacer on the stationary
10   phase and C_d is the concentration of displacer in the
mobile phase.

        Although applicants do not wish to be bound by
this hypothetical construct, it appears consistent
with the discovery that small molecules can be
15   effective displacers, because size is not the
critical parameter. According to the theory, any
molecule whose K_i and ν_i places it counterclockwise on
the affinity plot from the protein in question will
function as an effective displacer for that protein.

20   Consistent with this prediction, a number of low
molecular weight displacers have been tested and
found effective for protein displacement.

        Dendritic polymers (also known as starburst
polymers) are three-dimensional, highly ordered
25   oligomeric and polymeric compounds formed by
reiterative reaction sequences starting from smaller
molecules - "initiator cores" such as ammonia or
pentaerythritol. With selected building blocks and
propagation reactions, critical molecular design
30   parameters such as size, shape, topology, flexibility
and surface chemistry can be precisely controlled at

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the molecular level. The syntheses proceed via
discrete stages referred to as generations.
Dendrimers possess three distinguishing architectural
features: (1) an initiator-core region, (2) interior
5   zones containing cascading tiers of branch cells with
radial connectivity to the initiator core, and (3) an
exterior or surface region of terminal moieties
attached to the outermost generation.

The synthesis of a zero (12), first (14) and
10   second [(16) shown in scheme 2] generation
pentaerythritol based dendrimer was carried out as
described in detail later. The zero generation
dendrimer is referred to for convenience as PETMA4,
PentaErythrityl (TriMethylAmmonium)₄, the first
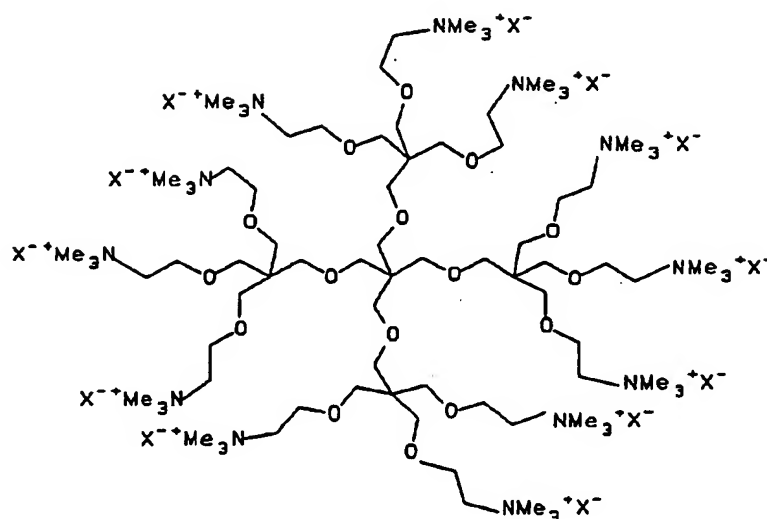15   generation as PETMA12, and the second as PETMA36.

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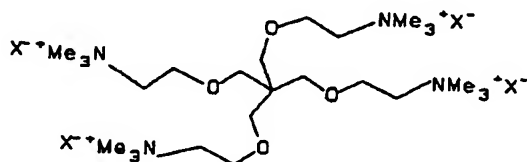
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The SMA model equilibrium parameters for the
zero, first and second generation dendrimers were
estimated in a 50 x 5 mm I.D. SCX column using
5   frontal chromatographic techniques.

As can be seen from Table 1, approximately
one-third of the total number of charges on each of
the dendrimers bind to the surface. The first
generation (PETMA12) and the second generation
10   (PETMA36) dendrimers exhibited similar adsorption
behavior, with similar values of σ_D/v_D and $Q_D * v_D$ and
marginal increases in Q_D with decrease in salt
concentration.

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TABLE 1
AVERAGE VALUES OF SMA PARAMETERS FOR PENTAERYTHRITOL BASED DENDRIMERIC DISPLACERS

Displacer (M.W.)	Salt (Na$^+$) Conc. (mM)	Solute Concen. C (mM)	Charac. Charge (v_D)	Steric Factor (σ_D)	(σ_D/v_D)	Q_D (mM)	$Q_D \cdot v_D$ (meq)
PETMA4 (480)	20	15	1.5	2.6	1.73	140	210
PETMA4 (480)	50	21	1.6	1.5	0.94	183	293
PETMA4 (480)	50	4.18	N.D.	N.D.	N.D.	177	N.D.
PETMA12 (1620)	20	6.17	4.2	6.3	1.50	55.3	232
PETMA12 (1620)	75	6.17	4.2	N.D.	N.D.	51.5	216
PETMA36 (5128)	20	1.96	11	16.7	1.52	20.9	230
PETMA36 (5128)	50	1.96	10.5	N.D.	N.D.	18.3	192

As seen in Table 1, the second generation dendrimer PETMA36 has a relatively higher characteristic charge than the first generation dendrimer, but a similar σ_i/v_i ratio. According to theory, PETMA36 should act as an efficient displacer, and that was indeed found to be the case. A two-protein displacement separation (α-chymotrypsinogen A and cytochrome C) using PETMA36 was carried out in a 100 x 5 mm cation exchange column. There was a reasonably good match of theory and experiment. The experiment was repeated using purified (diafiltered) first generation pentaerythritol PETMA12 as the displacer. These displacements indicate that decreasing the molecular weight and number of charged groups on the dendrimers appears to have little effect on their efficacy as displacers. Extending the prediction one level further, one would predict a zero generation dendrimer should also act as a

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protein displacer. This prediction runs counter to
the conventional wisdom of using high-molecular
weight polyelectrolytes with high characteristic
charges as displacers of proteins in ion-exchange
5   systems. (The zero generation dendrimer has a net
charge of 4, a characteristic charge of 1.7 and a
molecular weight of 480 Da.)

The results of the displacement chromatography
of the two-protein mixture of α-chymotrypsinogen A
10   and cytochrome-C with the zero generation dendritic
displacer are shown in Figure 6. As seen in the
figure, an excellent displacement separation of the
two proteins is observed in highly concentrated
adjacent zones with sharp boundaries and relatively
15   minimal mixing. This result is truly revolutionary,
and is of profound significance for implementation of
displacement chromatography for large-scale protein
separations.

It is seen that the zero, first and second
20   generation dendritic polyelectrolytes function as
efficient displacers of proteins in ion-exchange
systems. More significantly the ability of a low
molecular weight compound such as the 'zero'
generation dendrimer (M.W. 480) to displace
25   relatively high molecular weight proteins is quite
exciting in the current context of understanding
displacement phenomena. Since the molecular weight
and number of charged groups on the dendrimers appear
to have little effect on their efficacy as
30   displacers, it may be more advantageous to use a
'zero' generation dendrimer as a displacer. The
synthesis of these molecules is much easier and

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involves fewer steps; (hence they are cheaper). They have the additional advantage of easy separation from any feed component zones during post-displacement, size-based downstream processing.

5   Other low molecular weight electrolytes also appear to function effectively as displacers for proteins. For example, modified amino acids and charge-bearing antibiotics can be used as displacers. By modified it is meant that the amino acid is
10   altered so as to change it from amphoteric to either cationic (for cation exchange displacement chromatography) or anionic (for anion exchange chromatography). This is most conveniently accomplished by esterifying the carboxylate to
15   produce cationic species or acylating the amine to produce anionic species.

Displacers whose charge is derived from carboxylate tend not to be very effective anionic displacers because of their lower characteristic
20   charge at a pH commonly used in chromatography; as a result, they would have to have an extremely high equilibrium constant to fall counterclockwise from most proteins of interest on the affinity plot. For this reason, among amino acids, acylated taurine
25   derivatives are more likely candidates for anionic displacers.

Carboxyl-derivatized amino acids provide very effective cationic displacers. For example, carbobenzoxylysine methyl ester, benzoylarginine
30   ethyl ester (BAEE), arginine methyl ester and argininamide are all effective displacers in the

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       displacement chromatography of α-chymotrypsinogen and
cytochrome-C. The first two have a single, positive
charge; arginine methyl ester and amide have two
positive charges, and as a result, a higher affinity
5   for the stationary phase. The resolution of α-
chymotrypsinogen and cytochrome-C in isotacic
displacement is comparable to the resolution obtained
using high molecular weight displacers such as DEAE
dextran. An example of a displacement chromatogram
10   of α-chymotrypsinogen A and cytochrome C on an 8
micron strong cation exchange column using 45 mM
benzoyl arginine ethyl ester in a 50 mM salt solution
at pH 6.0 is shown in Figure 7. The modified amino
acid displacers can be purchased in a very pure form
15   at a cost which is a small fraction of the cost of
high molecular weight displacers. In addition, their
small size provides them with better transport
properties and faster kinetics.

       Many antibiotics have the virtue that they are
20   small enough to be removed easily if found in desired
protein fractions, but in addition, they can often be
advantageously left in the protein fraction. In
order to achieve the desired combination of high
characteristic charge and equilibrium constant, it
25   appears that antibiotics having one or more strongly
dissociating functionalities are particularly useful.
Such antibiotics include the streptomycins, which
have two guanidine functionalities. An example of a
displacement chromatogram of α-chymotrypsinogen A and
30   cytochrome C on an 8 micron strong cation exchange
column using 45 mM streptomycin sulfate (m.w. 581) in
a 30 mM salt solution at pH 6.0 is shown in Figure 8.

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The demonstration that aminoacid esters, dissociated antibiotics and zero generation dendrimers, all having molecular weights under 600, are highly effective displacers confirms that

5   molecular weights above 2000 are not necessary for displacers for protein chromatography. Indeed we have found no instance of a charged species of molecular weight below 2000 that did not work, as long as the characteristic charge and equilibrium

10   constant were such that the SMA analysis (shown in figure 5 and explained above) predicted efficacy.

A displacement separation of a two-component protein mixture was also carried out using crude PETMA (12) as the displacer in a 100 x 5 mm cation-

15   exchange column. Although the protein components were displaced and well separated in adjacent zones, the effluent profile exhibited similar characteristics to earlier displacements with impure DEAE-dextran displacers. Most strikingly, the

20   cytochrome-C zone was considerably less concentrated in relation to the α-chymotrypsinogen A zone. Apparently, impurities in the displacer contributed to the desorption of the proteins and depression of their isotherms. It is therefore believed

25   advantageous to purify the dendrimers.

One characteristic of dendrimers is that a variety of terminal moieties may reside on the surface of the dendrimer. The terminal groups can be readily converted to functionalities that provide the

30   potential for different utilities, and dendrimers possess a very high density of these terminal moieties which reside in the final exterior layer.
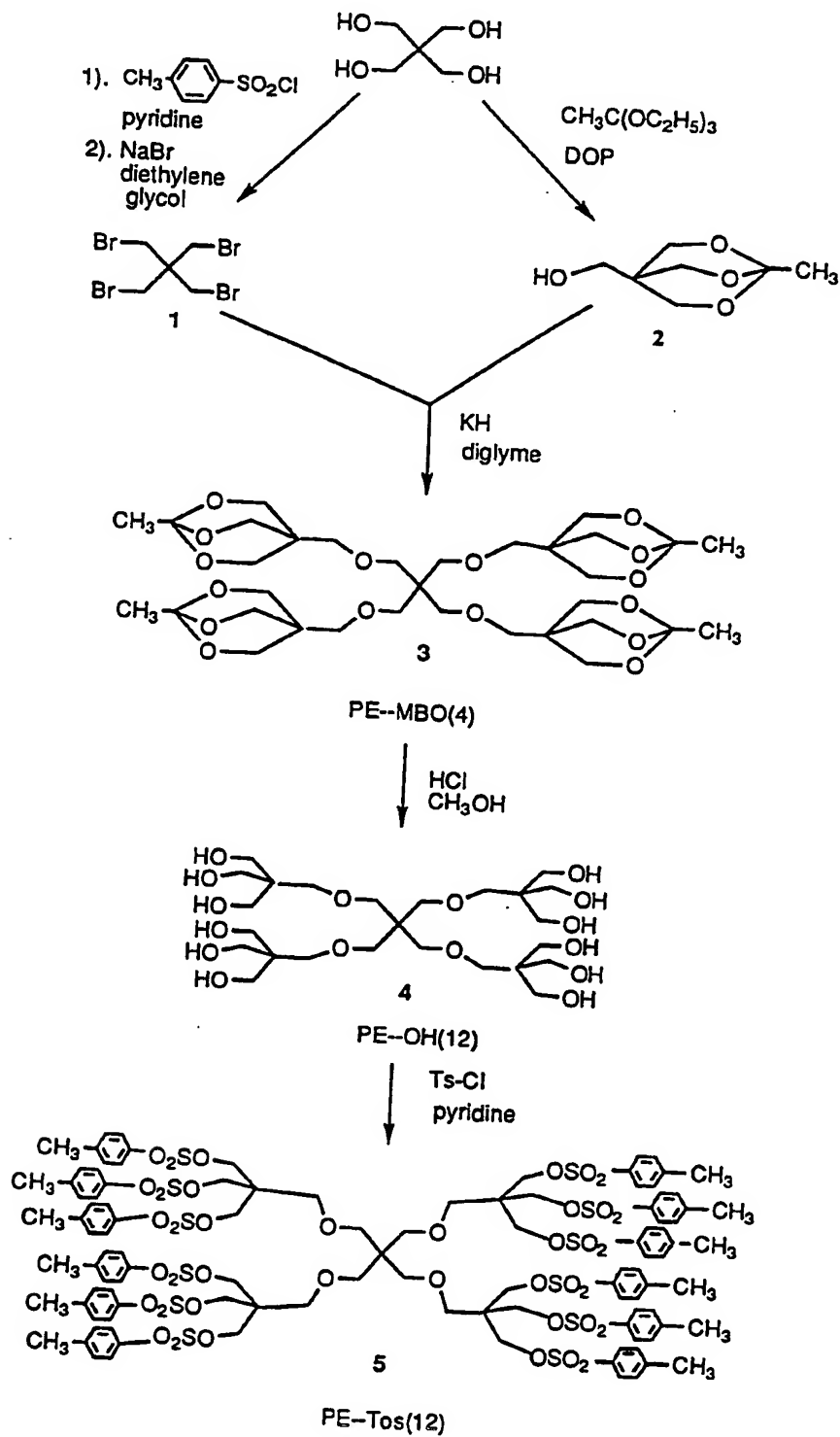
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When the organic groups on the surface of these
compact molecules are functionalized to be charged
groups, such as quaternary ammonium salts and
sulfonates, they exhibit higher affinity toward
5   chromatographic media than some proteins, making them
useful as a new type of displacer in chromatographic
separation. The synthesis of the backbone of a
dendritic polyether is shown in Scheme 1 and its
functionalization pathway to novel poly(ether-amine)s
10   is illustrated in Scheme 2. The functionalization of
dendrimeric precursors to provide anionic dendrimers
(sulfonates) is shown in Scheme 3. The strategy used
in Scheme 1 is a modified procedure derived from the
work of Hall and Padias [J. Org. Chem. 52, 5305
15   (1987)]. Pentaerythritol (PE) is also the initiator
core but 1-methyl-4-(hydroxymethyl)-2,6,7-
trioxabicyclo-[2.2.2]-octane (MHTBO) is used as the
building block instead of the hydroxymethyl bicyclic
orthoformate (HTBO). N,N-dimethylethanolamine is
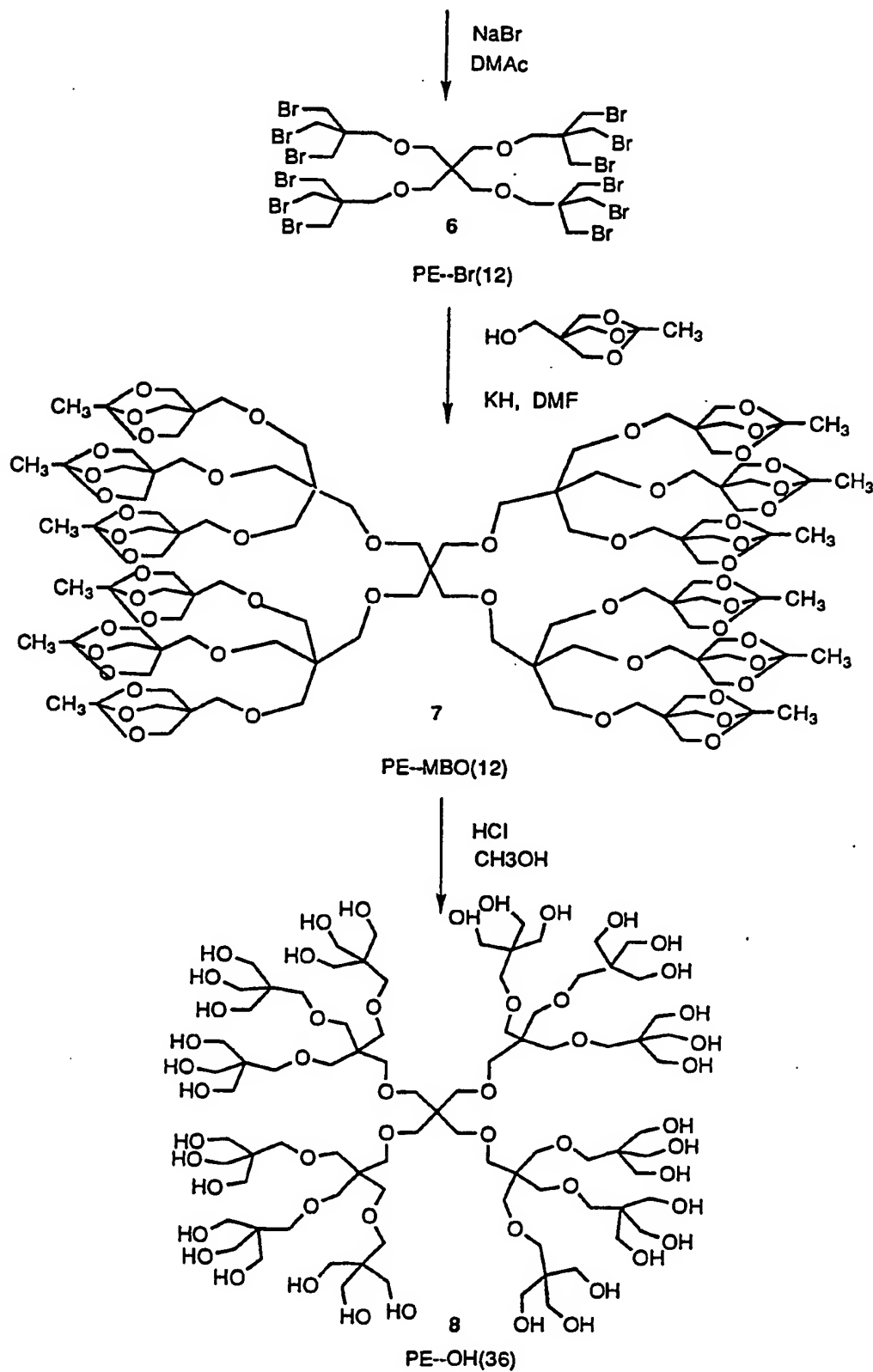20   used as the synthon to introduce tertiary amine
sites.

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SCHEME 1

PE--MBO(4)

PE--OH(12)

PE−Tos(12)

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SCHEME 1 (continued)

NaBr
DMAc

6

PE--Br(12)

KH, DMF

7

PE--MBO(12)

HCl
CH3OH

8

PE--OH(36)

-30-

SCHEME 1 (continued)
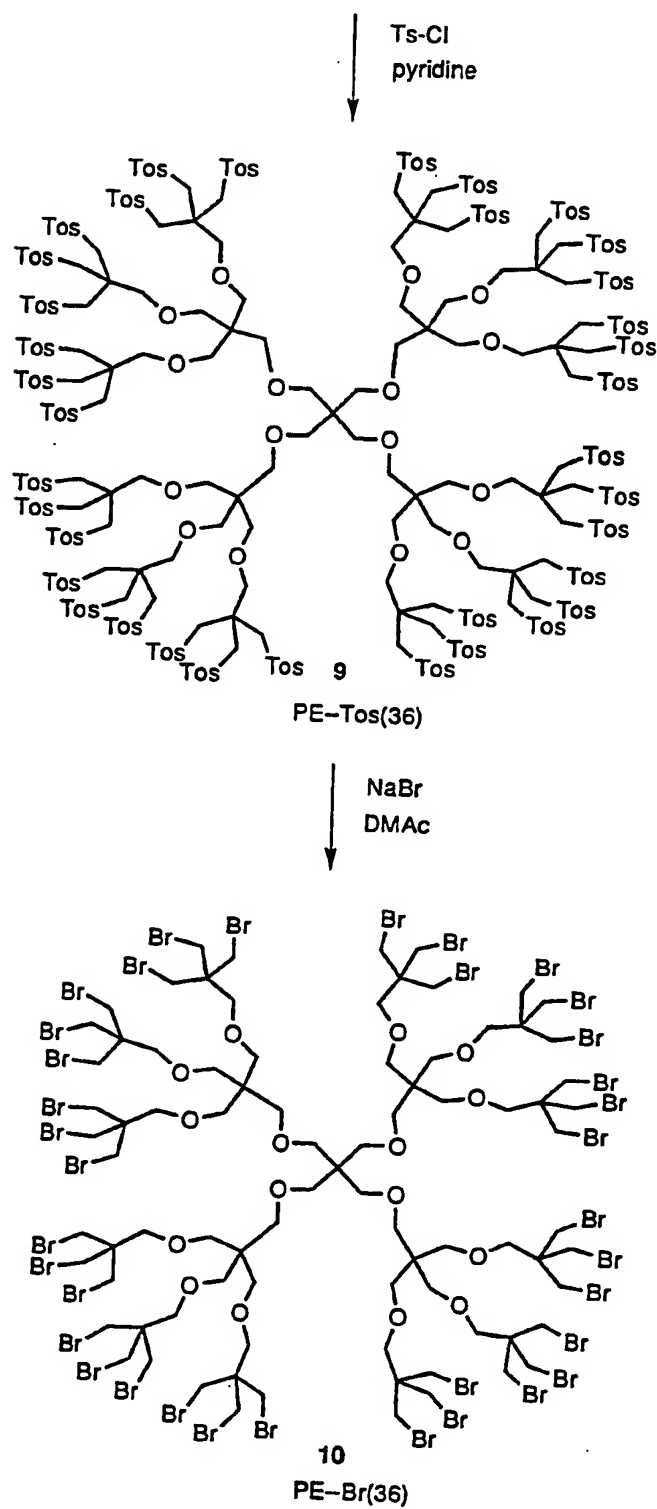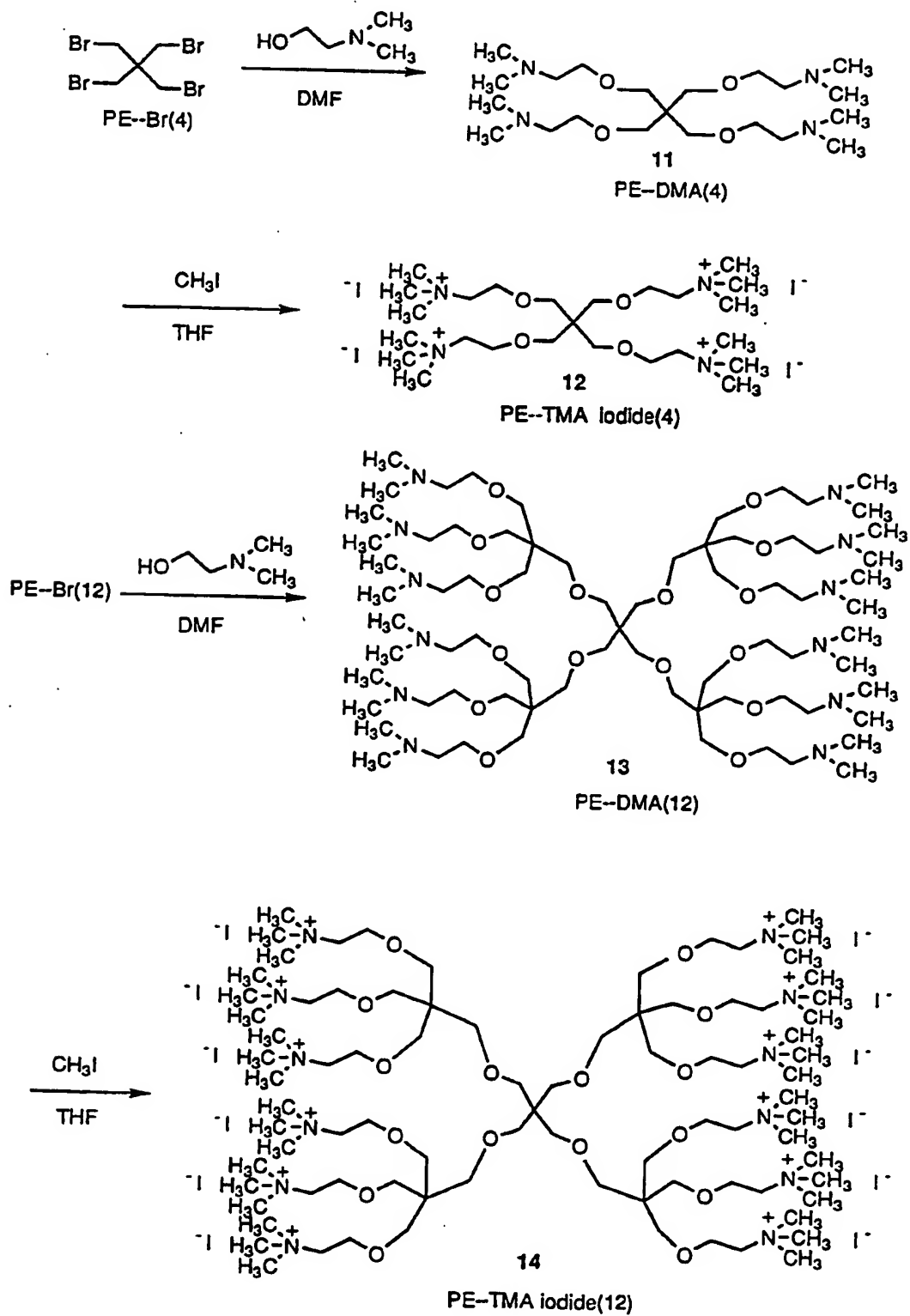
Ts-Cl
pyridine

9

PE–Tos(36)

NaBr
DMAc

10

PE–Br(36)

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SCHEME 2

11
PE–DMA(4)

12
PE--TMA iodide(4)

13
PE--DMA(12)

14
PE--TMA iodide(12)

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SCHEME 2 (continued)

PE--Br(36)

+

HO N CH₃ / CH₃

→ DMF

15

PE--DMA(36)

CH₃I / THF →

16

PE--TMA iodide(36)

−33−

SCHEME 3

17

PE--SPS(4)

18

PE--SPS(12)

19
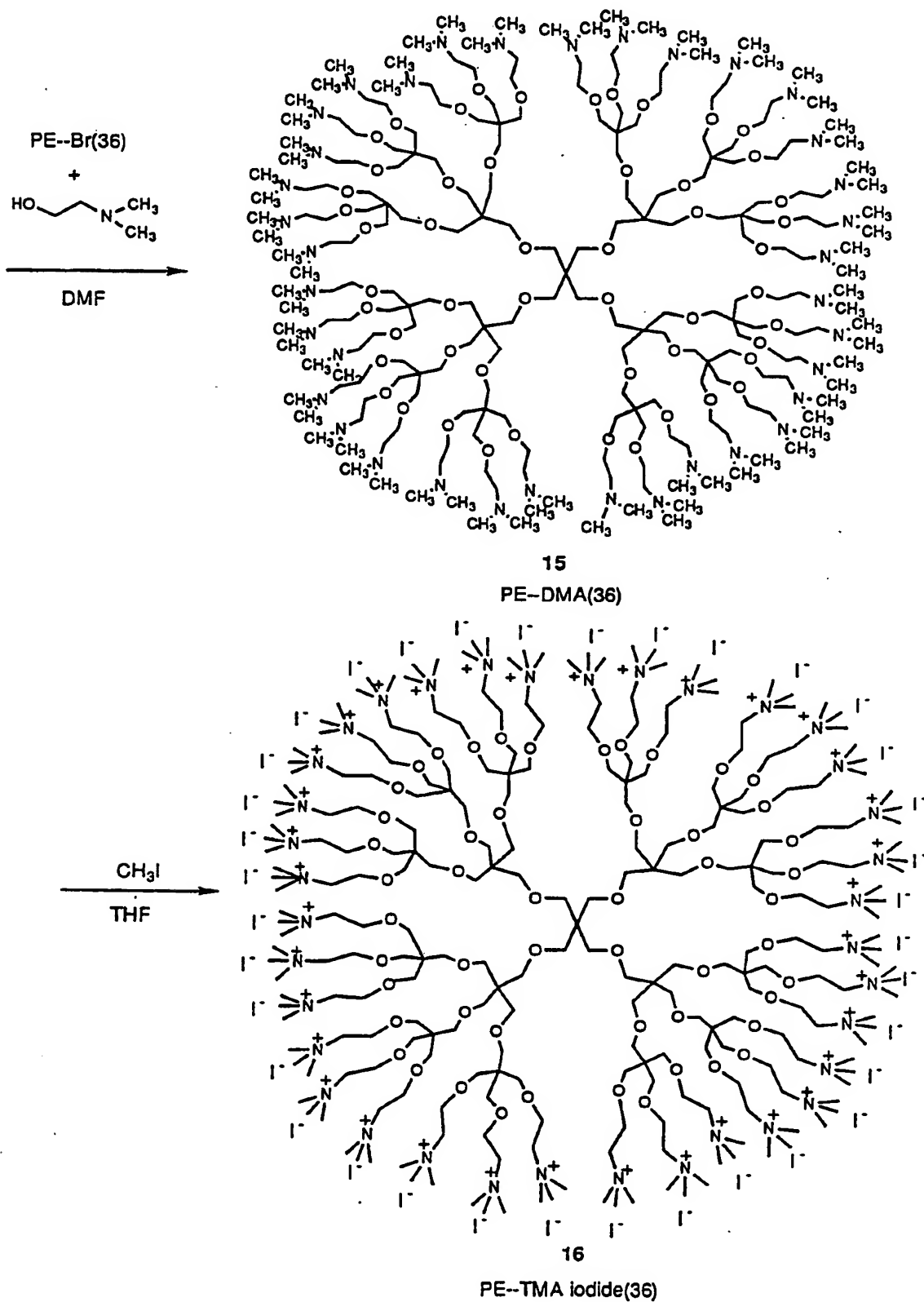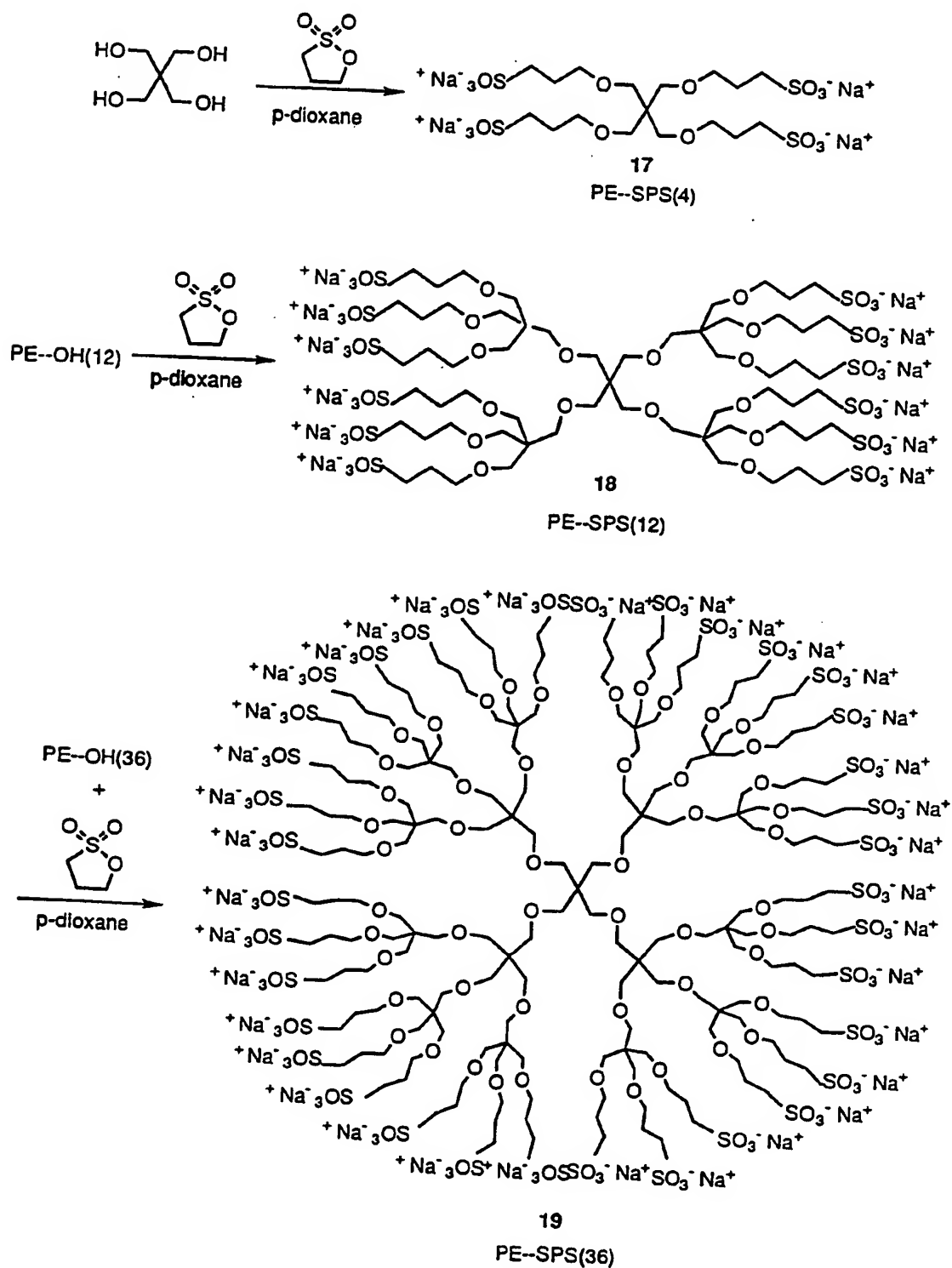
PE--SPS(36)

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Preparation of Dendrimeric Electrolytes

Hexane, tetrahydrofuran (THF), and 2-methoxyethyl ether (diglyme) were dried over sodium and distilled right before use. N,N-dimethyl-
5   formamide (DMF) was purchased from Aldrich Chemical Co. in HPLC grade. All other solvents and reagents were used without additional purification unless specified in the procedure.

Pentaerythrityl Tetrabromide, PE-Br(4) (compound 1)

10   In a 1 L, three-necked, round-bottom flask equipped with a mechanical stirrer and a thermometer were placed pentaerythritol (26.0 g. 0.19 mol) and 200 mL of pyridine. Stirring was initiated and to the suspension, cooled in an ice-bath, was added p-
15   toluenesulfonyl chloride (152.52g, 0.8 mol) as a solid at such a rate that the temperature did not rise above 30°C. After the addition was completed the resulting slurry was stirred at 35-40°C for another two hours. The slurry was then added slowly
20   to a vigorously stirred solution of 200 mL of water, 400 mL of methanol and 160 mL of concentrated hydrochloric acid. The crude white pentaerythrityl toluenesulfonate was further cooled by adding more ice, filtered with suction and washed with 1 L of
25   water and 200 mL of cold methanol in two portions.

In a 1 L, three-necked, round-bottom flask fitted with a mechanical stirrer, a thermometer and a condenser were mixed the slightly wet pentaerythrityl toluenesulfonate (about 140 g), sodium bromide (120
30   g, 1.16 mol) and 300 mL of diethylene glycol. The

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mixture was then heated to 140-150° C with slow
stirring and reacted in this temperature range
overnight. After being cooled to about room
temperature, the mixture was poured into 400 mL of
5   water with stirring, the precipitate was filtered
with suction and washed with 500 mL of water. The
crude product was dried under vacuum (1 torr) at 50°C
overnight and recrystallized from acetone. Yield:
52g (70%). Mp: 157-160° C.

10   **1-Methyl-4-(hydroxymethyl)-2,6,7-
trioxabicyclo[2.2.2]octane (MHTBO, compound 2)**

Pentaerythritol (13.6 g, 0.1 mol), triethyl
orthoacetate (16.22 g, 0.1 mol, 18.3 mL), pyridine p-
toluenesulfonate (PPTS) (0.5 g, 2 mmol) and 100 mL of
15   dioctyl phthalate were mixed in a 250 mL, round-
bottom flask fitted with a regular distillation
apparatus. The mixture was heated to 130-140°C and
ethanol was slowly distilled. When the amount of
ethanol was close to the theoretical value the
20   pressure was reduced to < 0.1 torr and the product
was distilled in vacuo. The white product which
distilled crystallized in the condenser to give 13.6-
14.9 g of MHTBO. Yield: 85-93%. The compound can
be recrystallized from toluene but can be used
25   directly. Mp: 110-112° C.

PE-MBO(4) (compound 3)

In a 500 mL, three-necked flask equipped with a
mechanical stirrer, a thermometer and an addition
funnel, under an argon atmosphere, potassium hydride
30   (2.8 g, 0.07 mol, 8.0 g of 35% suspension) was washed

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twice with hexane, the washings were decanted, and
100 mL of diglyme was added. After the mixture was
cooled to 0° C with stirring, a solution of 10.25 g
(0.064 mol) of MHTBO in 100 mL of diglyme was added
5   dropwise and the mixture was stirred at room
temperature for three hours. Then a solution of 5.82
g (0.015 mol) of pentaerythrityl tetrabromide in 100
mL of diglyme was added dropwise also at room
temperature. The mixture was heated to reflux for 24
10   hours. The mixture was then poured into 600 mL of
ice water and the precipitate was filtered, washed
with water, and dried under vacuum (1 torr) at 50° C
overnight. A white solid (8.2 g, 78%) was obtained.
The Beilstein test for bromide was negative. The
15   product was finally recrystallized from 4:1 ethyl
acetate/hexane. Yield: 5.86 g, 55%. Mp: 220° C
slight shrinkage, 230-244° C melting.

PE-OH(12) (compound 4)

In a 250 mL, round-bottom flask PE-MBO(4) (6.0
20   g, 8.53 mmol) was mixed with 100 mL of methanol and 1
mL of concentrated HCl. The mixture was heated
slowly to reflux and kept under reflux for one hour.
Methanol and methyl acetate were distilled off until
only about 1/3 of the solvent remained. The white
25   product was filtered and dried. Yield: 4.86 g (8.0
mmol), 94%. Mp: 180° C with shrinkage, 220-235° C
melting.

PE-Tos(12) (compound 5)

In a 500 mL Erlenmeyer flask PE-OH(12) (2.24 g
30   3.68 mmol) was dissolved in 40 mL of pyridine and

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cooled to 0° C. A solution of 21.2 g of p-toluenesulfonyl chloride (0.11 mol, 30 equiv) in 100 mL of pyridine was added dropwise through an addition funnel. The solution was stirred for another hour at

5   0° C and then left at room temperature for four days. The mixture was poured into 500mL of ice water and the solvent was decanted after the precipitate agglomerated at the bottom of the beaker. The crude product, 9 g, was dried under vacuum (1 torr) at 50°

10   C overnight and was then recrystallized from 4:1 ethanol/chloroform. Yield: 8.16 g (3.32 mmol), 90%. Mp: 130-133° C.

PE-Br(12) (compound 6)

PE-Tos (12) (8.0 g, 3.25 mmol) was dissolved in

15   50 mL of N,N-dimethylacetamide (DMAc) and 10.06 g of NaBr (98 mmol, 30 equiv) was then added. The resulting suspension was stirred and heated to 150° C and kept at this temperature for another hour. The mixture was cooled to room temperature and poured

20   into ice water. The precipitate was filtered, dried under vacuum (1 torr) overnight and recrystallized from ethyl acetate. Yield: 3.40 g (77%). Mp: 150° C with shrinkage, 172-177° C melting.

PE-MBO(12) (compound 7)

25   In a 500 mL, three-necked, round-bottom flask fitted with a mechanical stirrer, a thermometer and an addition funnel under an argon atmosphere potassium hydride (1.6 g, 40 mmol, 4.57 g of 35% suspension) was washed with hexane twice, the washes

30   were decanted and 100 mL of DMF was added. The

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mixture was cooled to 0° C and a solution of 5.76 g
of MHTBO (compound 2) (36 mmol, 24 equiv) in 50 mL of
DMF was added dropwise. The resulting suspension was
stirred for three hours at room temperature. The
5   dodecabromide (2.05 g, 1.5 mmol) was dissolved in 100
mL of DMF at 50° C and then was added rapidly
dropwise to the reaction flask immediately while
still warm. The mixture was heated to reflux for 24
hours and then poured into ice water containing about
10   50 g of sodium chloride. The precipitate was
filtered and dried under vacuum (1 torr) at 40° C,
overnight. The crude yield was quantitative (3.45 g)
and the product was purified by column chromatography
with silica gel as the stationary phase and a mixture
15   of 1:1 ethyl acetate/hexane as the eluent. R_f value:
0.42. Yield: 2.9 g (1.25 mmol), 82%. The Beilstein
test for halogen was negative. Mp: 78° C with
shrinkage, 88-90° C gelation, 170° C softening.

PE-OH(36) (compound 8)

20   In a 250 mL, round bottom flask were placed PE-
MBO(12) (4.4 g, 1.9 mmol), 100 mL of methanol and 1
mL of concentrated HCl. The mixture was heated to
reflux for one hour. Methanol and methyl acetate
were distilled until only 15-20 mL of the solution
25   remained and the solution was transferred to a
beaker. After the rest of the solvent was evaporated
completely, the syrup was dried under vacuum (1 torr)
to yield a foam. Yield: 3.35 g (1.66 mmol), 88%.
Mp: 75° C with shrinkage, 83-85° C gelation, 220-
30   230° C softening.

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PE-Tos (36) (compound 9)

In a 500 mL Erlenmeyer flask PE-OH(36) (4.17 g,
2.05 mmol) was dissolved in 180 mL of pyridine and
cooled to 0° C. A solution of p-toluenesulfonyl
5   chloride (29.4 g, 0.15 mol, 75 equiv) in 200 mL of
pyridine was added dropwise. The mixture was stirred
for another hour at 0° C and then left at room
temperature for 7 days. The brown solution was
poured into 2L of ice water and the precipitate was
10   filtered and dried under vacuum (1 torr) at 40° C
overnight. Yield: 14.36 g (1.88 mol), 92%. Mp:
120° C with shrinkage, 220-245° C melting.

PE-Br(36) (compound 10)

In a 250 mL, three-necked, round bottom flask
15   were mixed 7.58 g (1.0 mmol) of PE-Tos(36), 8.32 g
(80 mmol, 80 equiv) of NaBr and 100 mL of DMAc. The
mixture was stirred and heated to 150° C and kept at
this temperature for one hour. Then the mixture was
cooled to room temperature and poured into 2 L of ice
20   water with stirring. The precipitate was filtered,
washed with another 500 mL of water and dried under
vacuum (1 torr) at room temperature overnight.
Yield: 4.18 g, 98%. Mp: 48° C with shrinkage. 52-
68° C melting.

25   **Tetrakis [((N,N dimethylamino)ethoxy)methyl] methane,
PE-DMA(4) (compound 11)**

In a 500 mL, three necked, round-bottom flask
equipped with a mechanical stirrer, an addition
funnel and a thermometer, potassium hydride (5.2 g,

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0.13 mol, 14.8 g of a 35% suspension) was washed with
hexane twice under an argon atmosphere and 100 mL of
DMF was added. When the mixture was cooled to 0° C a
solution of 10.7 g (0.12 mol, 6 equiv) of N,N-
5   dimethylethanolamine in 100 mL of DMF was added
dropwise and stirred at room temperature for three
hours. A solution of 7.8 g (0.02 mol) of
pentaerythrityl tetrabromide in 100 mL of DMF was
added dropwise. The mixture was heated to 80° C and
10   reacted at 80-90° C for 12 hours. Then the
temperature was raised to reflux for another 12
hours. The resulting mixture was cooled to below 50°
C and poured into 300 mL of ice water. All of the
solvents were removed on a rotavapor and the residue
15   was extracted with 800 mL of ethyl ether in a few
portions, and the combined extracts were dried over
$MgSO_4$. After the ether was evaporated, the crude
product was distilled under vacuum (<0.1 torr) to
obtain 5.7 g of oily liquid PE-DMA(4). The compound
20   was further purified to a clear liquid on an Al_2O_3
column using a mixture of 4:1 ethyl acetate/hexane as
the eluent. R_f value: 0.57. Yield: 4.66 g 59%.
Bp: 140-143° C (0.03 mmHg).

Tetrakis [((N,N,N-trimethylammonium iodide)
25   ethoxy)methyl] methane, PE-TMA iodide (4)
(compound 12)

In a 100mL, three necked flask were placed PE-
DMA(4) (2.3 g, 5.5 mmol) and 30 mL of THF under an
argon atmosphere. The solution was cooled to 0° C
30   and a solution of CH_3I (9.3 g, 66 mmol, 12 equiv, 4.1
mL) in 20 mL of THF was added dropwise. After the
addition was completed the mixture was stirred at

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room temperature for five more hours. The yellowish
precipitate was filtered, dried under vacuum (1 torr)
at 60° C overnight and finally recrystallized from
methanol. The compound is highly hygroscopic.
5   Yield: 3.84 g, 70%.

PE-DMA(12) (compound 13)

The procedure is similar to the one used for PE-
DMA(4). Potassium hydride (2.8 g, 0.07 mol, 8.0 g of
a 35% suspension) was washed with hexane twice under
10   an argon atmosphere and 100 mL of DMF was added. A
solution of 6 mL (0.06 mol, 5.34 g) of N,N-
dimethylethanolamine in 50 mL of DMF was added
dropwise at 0° C and the mixture was stirred at room
temperature for three hours. PE-Br(12) (2.72g, 32
15   mmol) was dissolved in 150 mL of DMF at 50° C and the
solution was added while still warm through an
addition funnel, dropwise. The mixture was heated to
reflux for 24 hours and poured into 300 mL of ice
water. After all the DMF and water were evaporated,
20   the residue was extracted with 800 mL of ether in
several portions and dried over $MgSO_4$. The ether
solution was filtered and concentrated to about 200
mL. In a 300 mL, three necked flask, HCl gas was
introduced to the solution and the solvent was
25   decanted when no more salt was formed. The salt was
washed with anhydrous ether twice, dried under argon
for half an hour, dissolved in water and basified to
pH>10. The resulting aqueous solution was dried on a
rotavapor and the residue was extracted with 500 mL
30   of ether and dried over $MgSO_4$. After the ether was
evaporated, 2.1 g of fairly pure product was
obtained. The compound is a viscous oil; bp 220° C

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at 0.02 torr. Yield: 72%.

PE-TMA iodide (12) (compound 14)

      The procedure is identical with the one used for
PE-TMA (iodide(4)). PE-DMA (12) (2.04 g 1.4 mmol) was
5   dissolved in 60 mL of THF. At 0° C a solution of 3.2
mL (7.12 g, 50 mmol, 36 equiv.) of CH_3I in 20 mL THF
was added dropwise. The mixture was stirred at room
temperature for another three hours. The yellow
precipitate was filtered and dried under vacuum (1
10   torr) at 60° C overnight. This salt cannot be
recrystallized from methanol and was further purified
by ultrafiltration before being tested as a
displacer. Yield: 3.74 g, 84%.

PE-DMA(36) (compound 15)

15   This procedure is identical with the one used
for PE-DMA(12). Potassium hydride (2.8 g, 0.07 mmol,
8.0 g of 35% suspension) was washed twice with hexane
and 100 mL of DMF was added. At 0° C a solution of
6.4 mL (5.7 g, 64 mmol, 80 equiv.) of N,N-
20   dimethylethanolamine in 50 mL of DMF was added and
the mixture was stirred at room temperature for three
hours. PE-Br(36) (3.43 g, 0.8 mmol) was dissolved in
100 mL of DMF and added dropwise at room temperature.
The mixture was then heated to reflux for 24 hours
25   and poured into 200 mL of ice water. After all
solvents were removed, the residue was extracted with
800 mL of ether. The polyamine was converted to a
salt by bubbling HCl gas into an ether solution and
then was freed by basifying the aqueous solution to
30   pH>10. This compound is a very viscous syrup and is

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highly hygroscopic. Yield: 1.86 g, 51%.

PE-TMA iodide(36) (compound 16)

The procedure is also identical to the one used for PE-TMA iodide(4) and PE-TMA(12). PE-DMA(36)
5   (1.79 g, 0.39 mmol) was dissolved in 100 mL of THF and cooled to 0° C. A solution of 4.2 g (1.82 mL, 30 mmol, 75 equiv.) of CH_3I in 20 mL of THF was added and the mixture was stirred at room temperature for another three hours. The precipitate was then
10   filtered and dried under vacuum (1 torr) at 60° C overnight. Yield: 2.8 g, 75%.

While the invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that other changes in form and details may be made therein without departing from the spirit and scope of the invention.

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Claims

1.   A method for purifying a protein comprising
loading said protein in a suitable loading solvent
onto an ion exchange stationary phase and displacing
said protein from said stationary phase by
5   displacement chromatography using a displacer of
molecular weight less than 1620.

2.   A method according to claim 1 wherein said
stationary phase is a cation exchange resin and said
displacer is a cationic species.

3.   A method according to claim 2 wherein said
displacer is a poly(quaternary ammonium) salt.

4.   A method according to claim 2 wherein said
displacer is
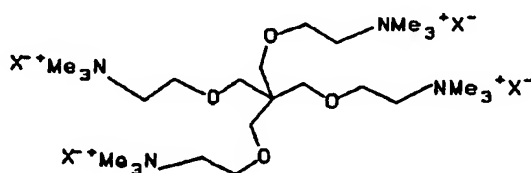
wherein X is a counter anion selected from the group
consisting of halogen, sulfate, sulfonate,
5   perchlorate, acetate, phosphate and nitrate.

5.   A method according to claim 1 wherein said
stationary phase is an anion exchange resin and said
displacer is an anionic species.

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6.   A method according to claim 1 wherein said
displacer is selected from the group consisting of
aminoacid esters, aminoacid amides, N-acylaminoacids,
peptide esters, peptide amides and N-acyl peptides.

7.   A method according to claim 6 wherein said
displacer is selected from the group consisting of
lower alkyl esters and amides of lysine, lower alkyl
esters and amides of arginine, lower alkyl esters and

5   amides of N^{α}-acylated lysine and lower alkyl esters
and amides of N^{α}-acylated arginine.

8.   A method according to claim 1 wherein said
displacer is a dendritic polymer.

9.   A method according to claim 1 wherein said
displacer is a cationic antibiotic.

10.   A method according to claim 1 wherein said
displacer is an anionic antibiotic.

11.   A method according to claim 1 wherein said
displacer is dissolved in a solvent system and said
displacer is selected from electrolytes whose
characteristic charge (ν) and equilibrium constant

5   (K) are such that when a coordinate system
representing logK on the ordinate and ν on the
abscissa is created, a line constructed from a point
A on the ordinate axis through a point defined by the
K and the ν of the displacer has a greater slope than

10   a corresponding line constructed from the same point
A   through a point defined by the K and the ν of the
protein to be purified, said point A corresponding in
value to the slope of the displacer operating line

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(Δ) in said solvent system in which said displacer is
15   dissolved.

      12.   A method for purifying a protein comprising
loading said protein in a suitable loading solvent
onto an ion exchange stationary phase and displacing
said protein from said stationary phase by
5   displacement chromatography using a dendritic
polyelectrolyte displacer.

      13.   A method according to claim 12 wherein said
stationary phase is a cation exchange resin and said
polyelectrolyte displacer is a polycation.

      14.   A method according to claim 13 wherein said
polyelectrolyte displacer is a poly(quaternary
ammonium) salt.

      15.   A method according to claim 14 wherein
said displacer is
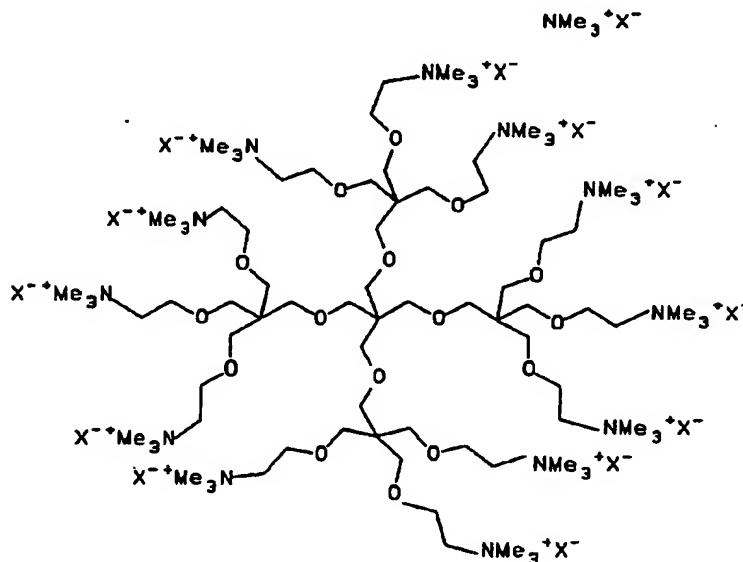
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wherein X⁻ is a counter anion selected from the group consisting of halogen, sulfate, sulfonate, perchlorate, acetate, phosphate and nitrate.

16.   A compound of formula
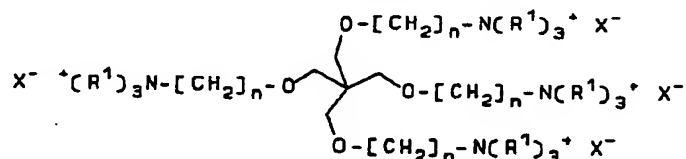
wherein R^1 is lower alkyl, n is 2 to 6, and X is halogen, sulfate, sulfonate, perchlorate, acetate, phosphate or nitrate.
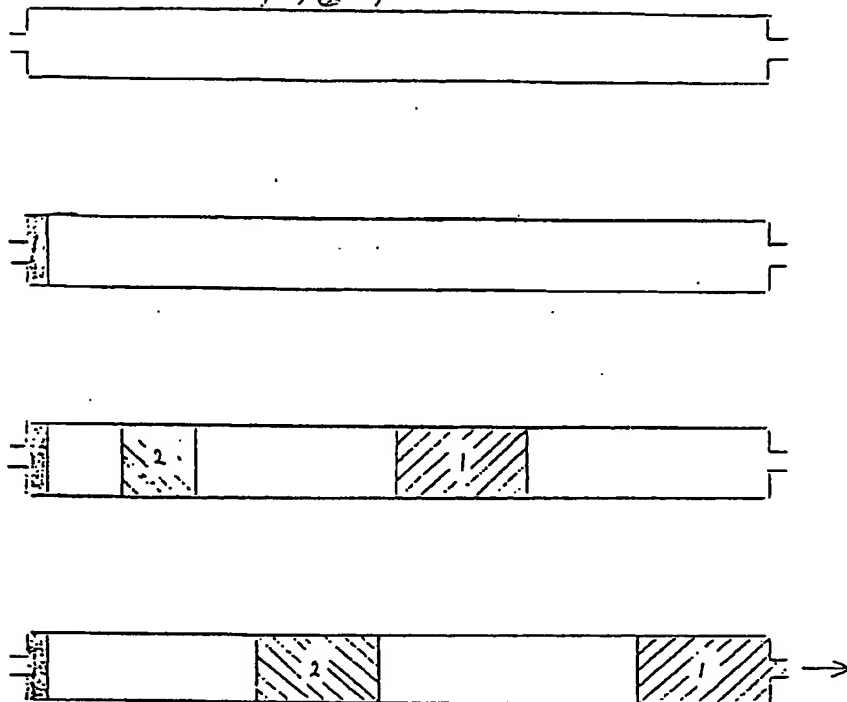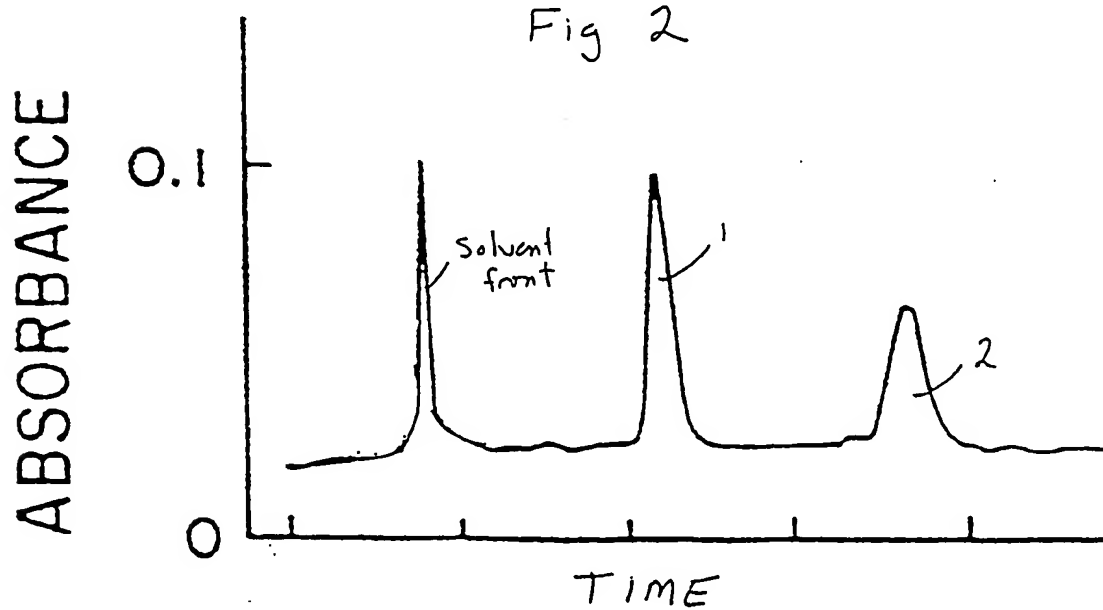
17.   A compound of formula
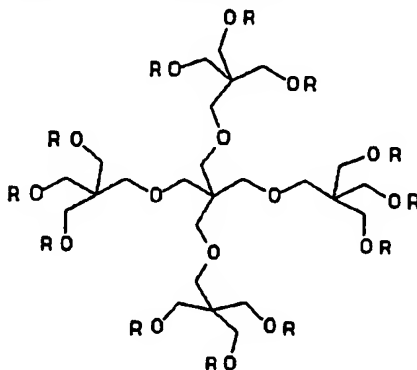
wherein R is $-(CH_2)_n-N(R^1)_3^+$ X⁻, R^1 is lower alkyl, n is 2 to 6, and X is halogen, sulfate, sulfonate, perchlorate, acetate, phosphate or nitrate.

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FIG 1

Fig 2

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FIG 3

Carrier
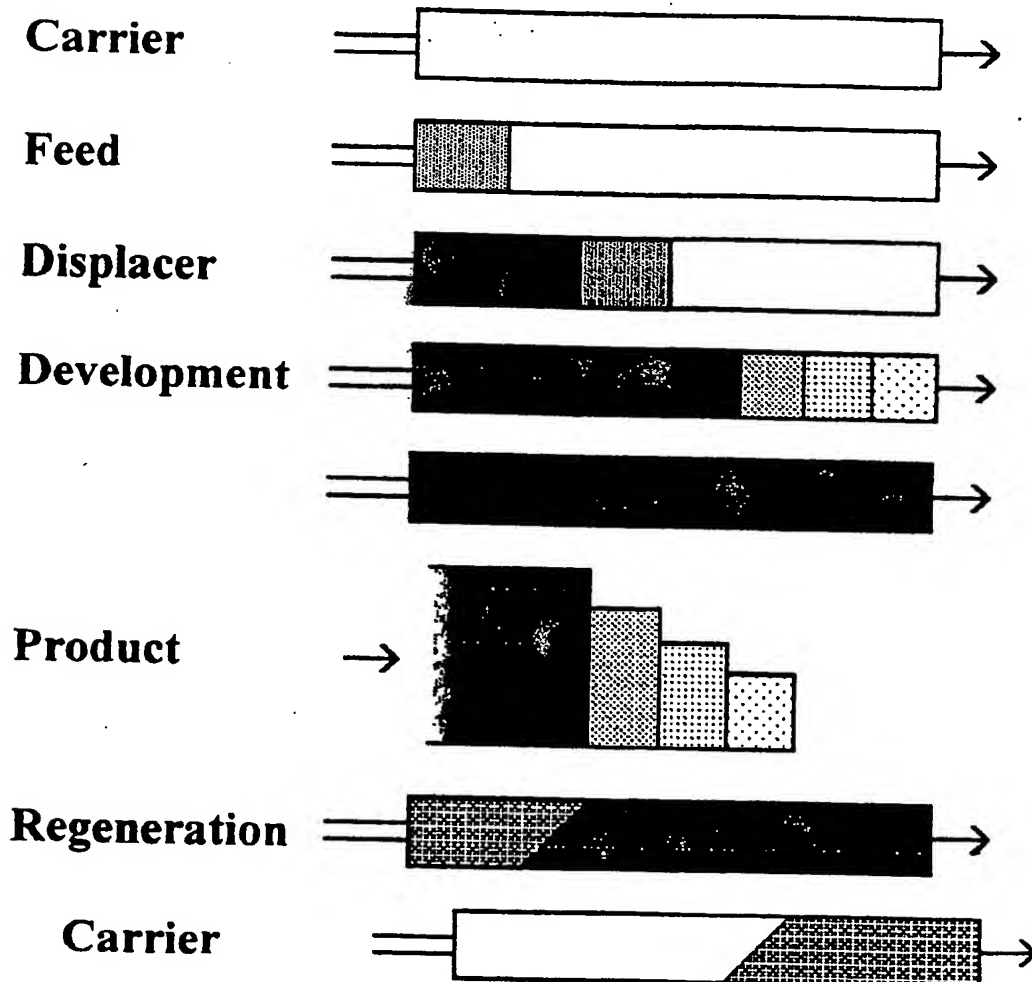
Feed

Displacer

Development

Product

Regeneration

Carrier

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Fig 4

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Fig 5

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Fig 6

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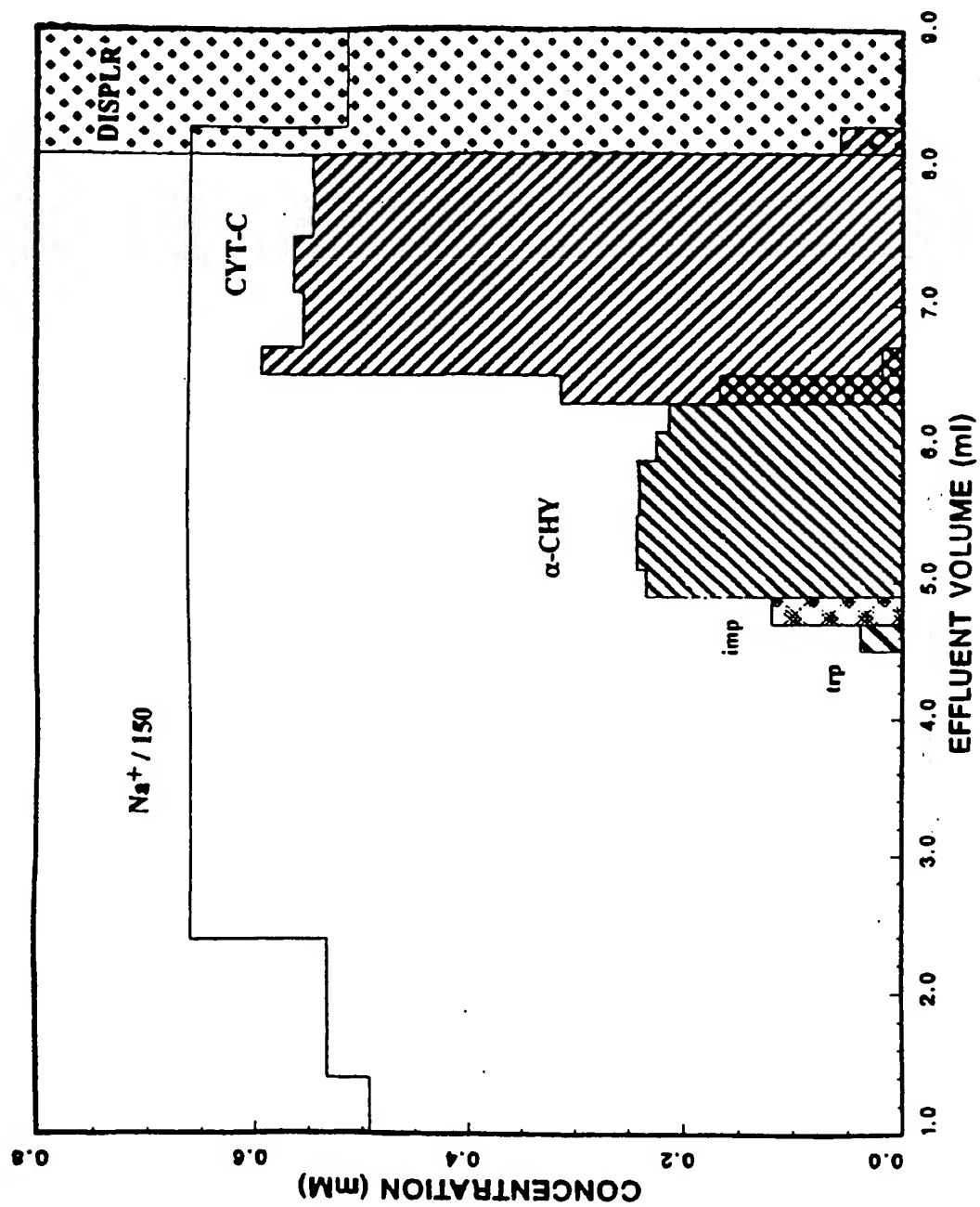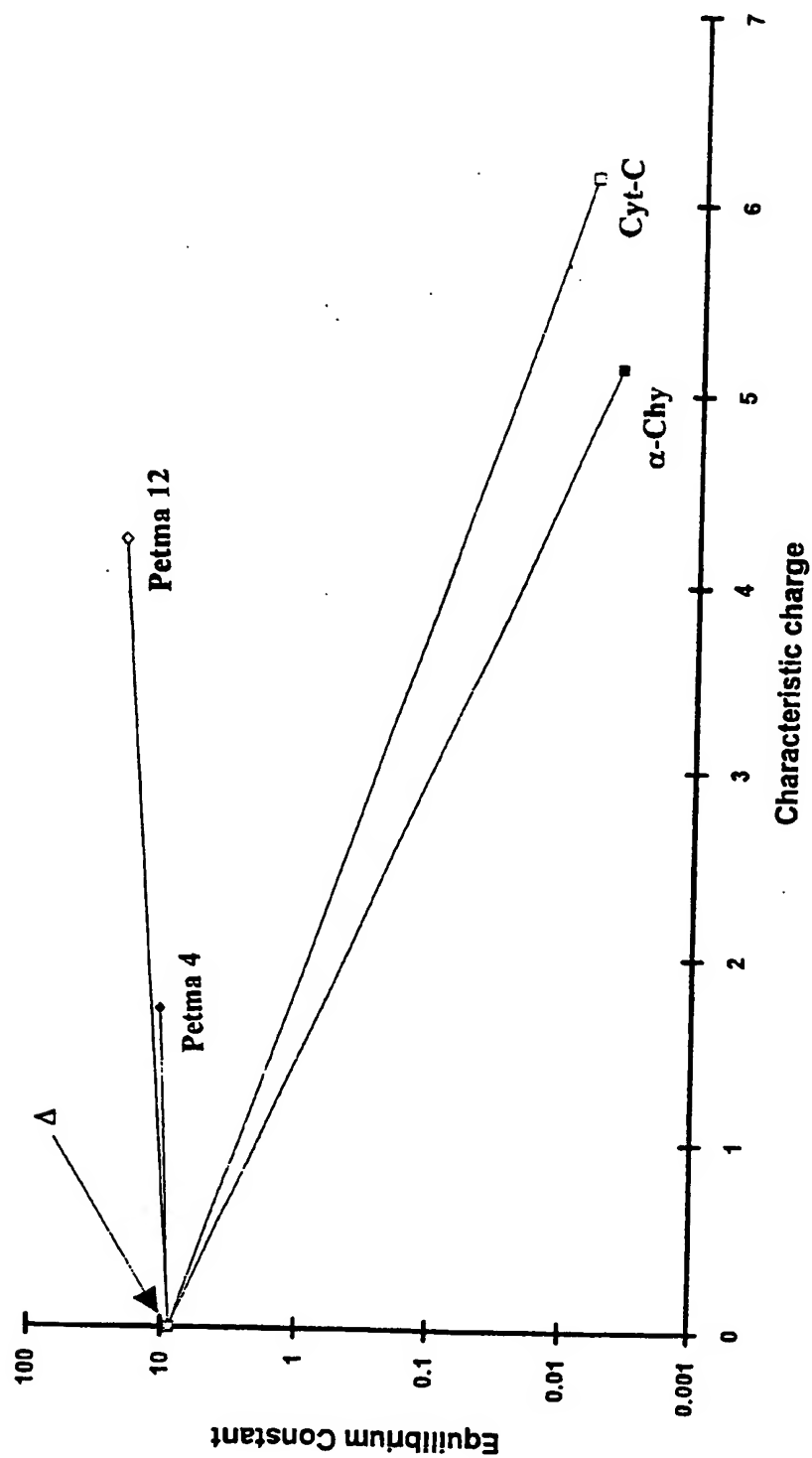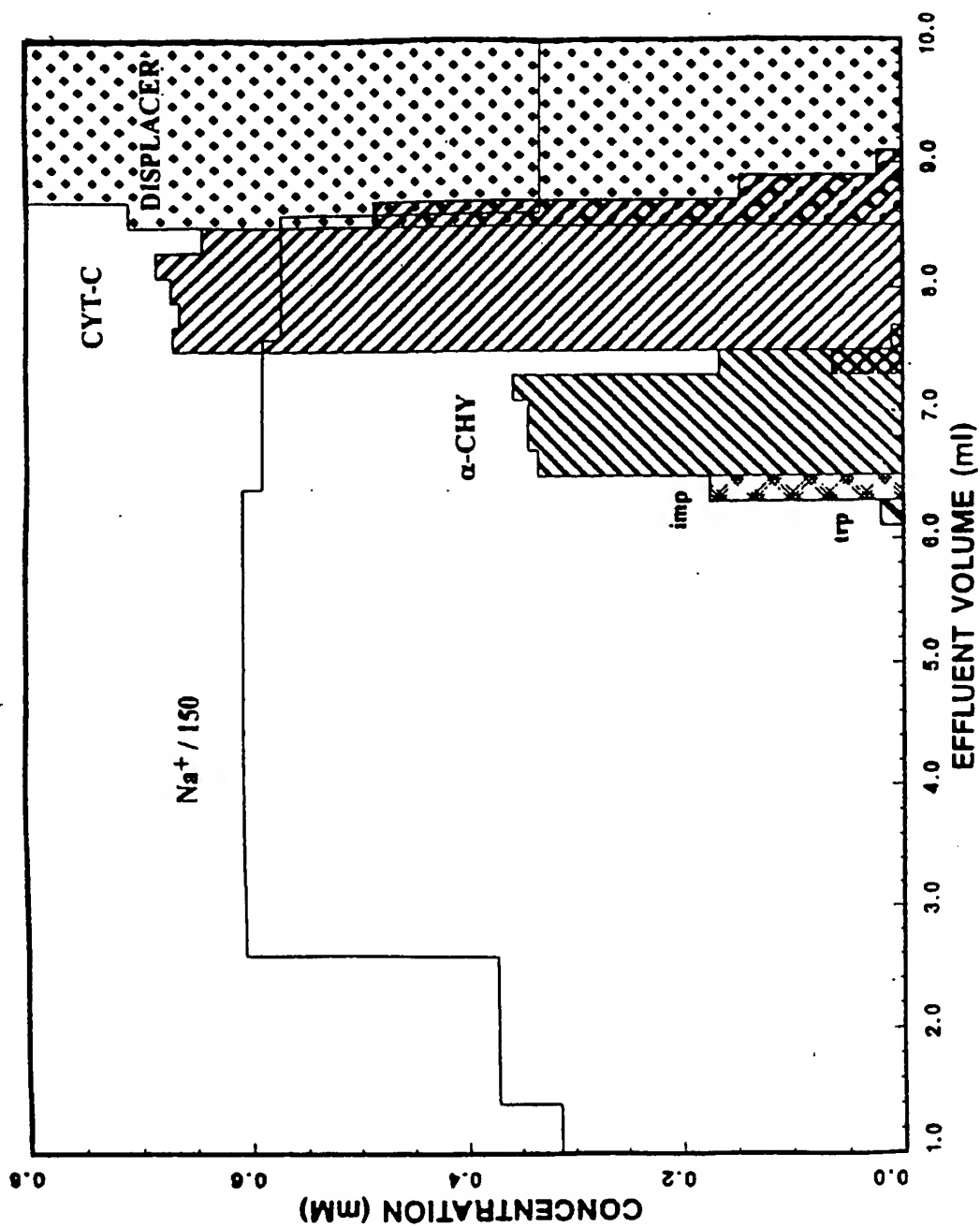
Fig 7
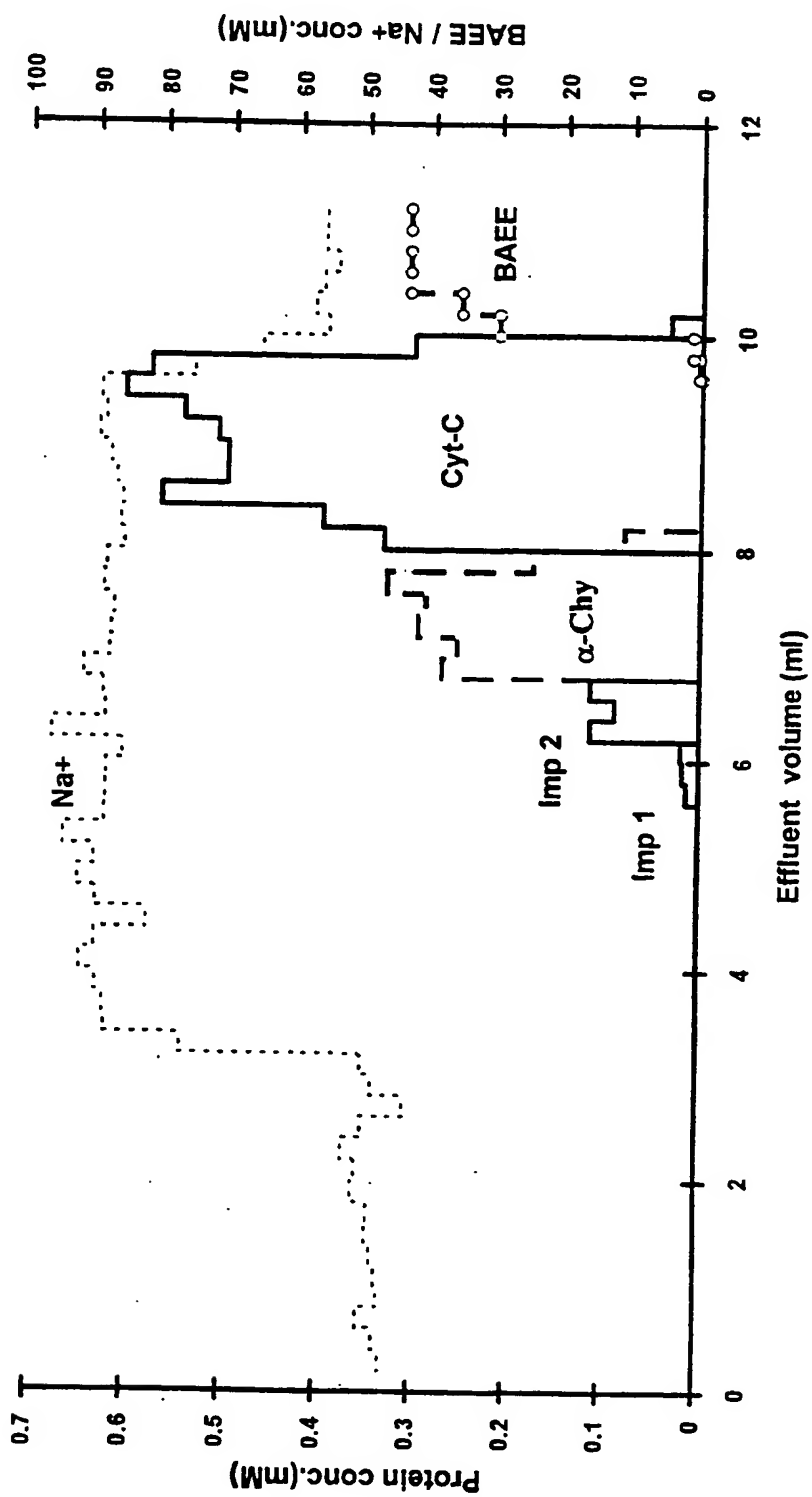
7/7

Fig 8
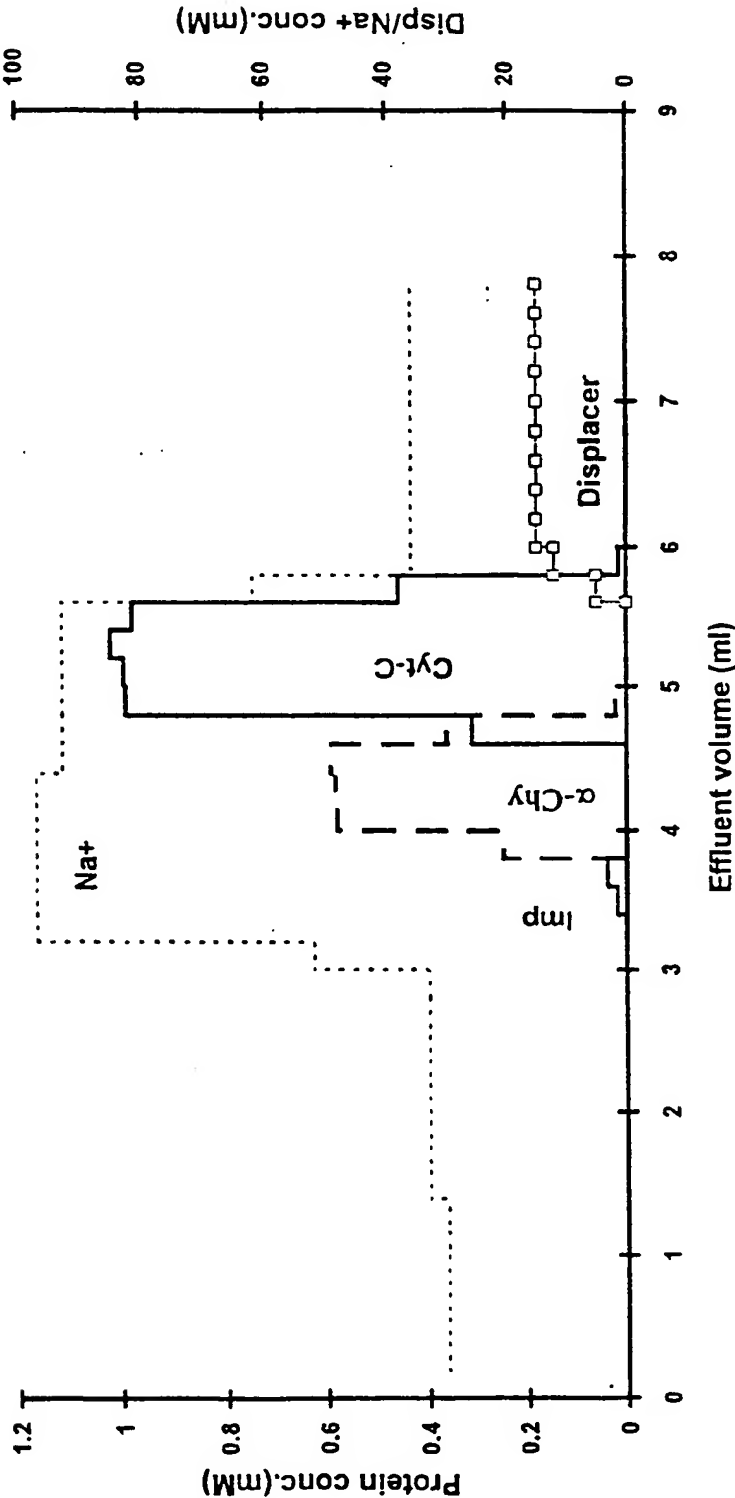
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01966

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 01/14, 01/16, 01/18; C07C 211/62
US CL :530/344, 412, 415, 416; 564/292, 294, 295
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/344, 412, 415, 416; 564/292, 294, 295

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, DERWENT

search terms: displac? chromtog?, ion? anion? cation? exchang?, quartenary ammonium

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Chromatography, Volume 519, issued 1990, S.-C. David Jen et al, "Use of the Sodium Salt of Poly(vinylsulfonic acid) as a Low-Molecular Weight Displacer for Protein Separation by Ion-Exchange Displacement Chromatography", pages 87-98, see page 87, abstract, lines 1-11, page 88, lines 31-33 and lines 44-49 through page 89 line 1 and Table I.	1, 5
A	Makromolecular Chemistry, Volume 181, issued 1980, H. Eckstein et al, "Synthese Vernetzter Lysin- und Lysinpeptidgele", pages 2471-2480, see the summary.	1-17

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 MARCH 1995	2 6 MAY 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	NANCY J. DEGEN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01966

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Organic Chemistry, Volume 50, issued 1985, G. R. Newkome et al, "Cascade Molecules: A New Approach to Micelles. A [27]-Arborol", pages 2003-2004, see entire document.	1-17
A	Chemical Abstracts, Volume 105, issued 1986, I. Imai et al, "Photocurable Coating Compositions", see abstract number 154826.	1-17
A	E. HEFTMANN, "CHROMATOGRAPHY. Fundamentals and applications of chromatographic and electrophoretic methods. Part A: Fundamentals and Techniques", published 1983 by Elsvier Scientific Publishing Company (N. Y.), pages A30-A33.	1-17
A	US, A, 5,156,835 (NABI ET AL) 20 OCTOBER 1992, see entire document.	1-17
A	US, A, 5,043,423 (VISCOMI ET AL) 27 AUGUST 1991, see entire document.	1-17
A	US, A, 5,028,696 (TORRES ET AL) 02 JULY 1991, see entire document.	1-17

INTERNATIONAL SEARCH REPORT

B. FIELDS SEARCHED

Documentation other than minimum documentation that are included in the fields searched:

CHROMATOGRAPHY, VOLS. A & B, HEFTMAN, 1983; HIGH PRESSURE LIQUID CHROMATOGRAPHY, HORVATH, 1983; CHROMATOGRAPHIC METHODS, STOCK & RICE, 1974; INTRO. TO MODERN CHROMATOGRAPHY, SNYDER & KIRKLAND, 1979

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